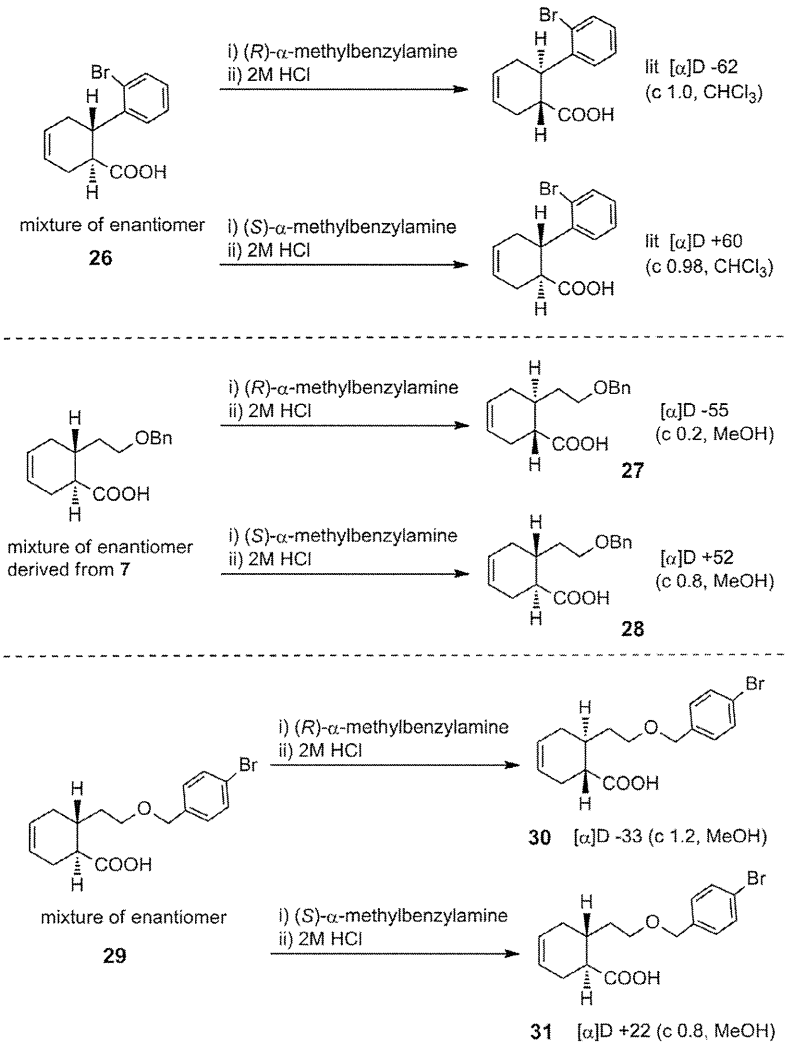


Scheme 4. Resolution by salt formation.

conformational differences at the *N*-substituent, as well as the interactions at the S_2 pocket, explain the discrepancy in the inhibitory activity between (4a*R*,8a*S*) and (4a*S*,8a*R*) decahydroisoquinolin inhibitors (**41** vs **44**).

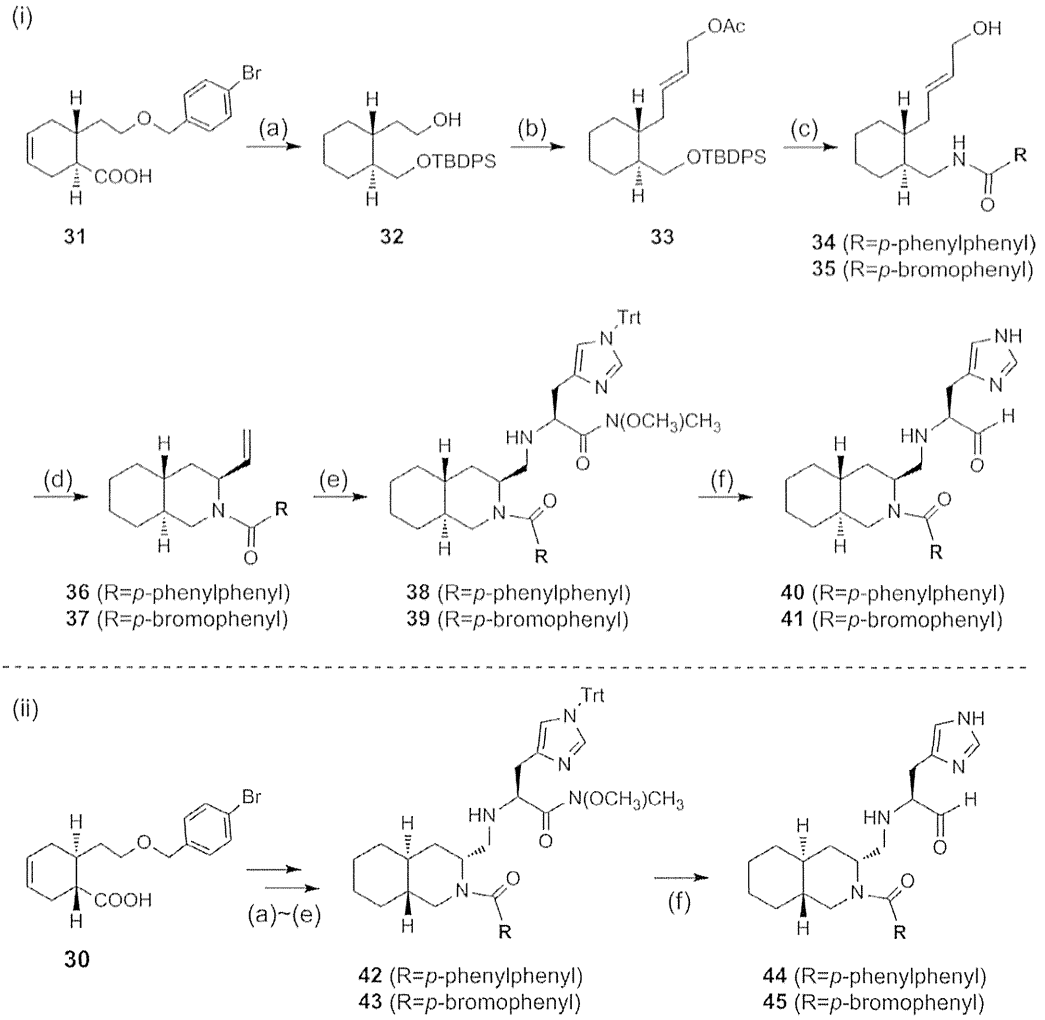
3. Conclusion

A novel non-peptide inhibitor based on the interactions at the S_1 and S_2 sites of SARS $3CL^{pro}$ was designed and synthesized. Focusing on cleavage site interaction at the S_1 site and hydrophobic interaction at the S_2 site, a decahydroisoquinolin scaffold was designed. Using a cyclohexene enantiomer obtained by salt resolution using chiral amine, the *trans*-decahydroisoquinolin derivative was synthesized as an enantiomer. Several analogs containing different *N*-substituents were also prepared similarly. All decahydroisoquinolin inhibitors showed moderate but clear inhibitory activities for SARS $3CL^{pro}$, which confirmed that the fused ring structure of the decahydroisoquinolin scaffold functions as an inhibitor for SARS $3CL^{pro}$. By X-ray crystallographic studies, it was confirmed that the decahydroisoquinolin inhibitors were at the active site cleft of $3CL^{pro}$, as observed in the highly potent peptide–aldehyde inhibitor. The decahydroisoquinolin scaffold was inserted into a large S_2 pocket and occupied most of the pocket. The P_1 site imidazole was inserted into the S_1 pocket as expected. These interactions were effective to hold the terminal aldehyde tightly inside the active site cleft, which resulted in the compact fitting of the novel scaffold to $3CL^{pro}$. The acyl substituent on the nitrogen in the decahydroisoquinolin scaffold was at the surface of the $3CL^{pro}$, where additional interactions with the $3CL^{pro}$ may be possible. Evaluations on the analogs focusing on the interactions at the *N*-substituent are now underway.

4. Experimental

4.1. General

All solvents were of reagent grade. THF was distilled from sodium and benzophenone ketyl. CH_2Cl_2 was distilled from CaH_2. All commercial reagents were of the highest purity available. Analytical TLC was performed on silica gel (60 F-254, 0.25 mm Plates). Column chromatography was carried out on Wakogel C-200E (particle size, 75–150 μm) or Wakogel FC-40 (particle size, 20–40 μm). ^1H NMR spectra were recorded in $CDCl_3$ (unless otherwise stated) on agilent UNITY INOVA 400 NB, JEOL JNM-ECS 400, Bruker AM-300, or JEOL JNM-LA 500 spectrometers. Chemical shifts are expressed in ppm relative to tetramethylsilane (0 ppm) or $CHCl_3$ (7.28 ppm). The coupling constants are given in Hz. ^{13}C NMR spectra were recorded on the same spectrometers at 100 or 125 MHz, using the central resonance of $CDCl_3$ (δ_C 77.0 ppm) as the internal reference unless otherwise stated. High-resolution mass spectra



Scheme 5. Construction of the decahydroisoquinolin scaffold starting from the separated enantiomer. Reagents: (a) (1) IBCF/$NaBH_4$, (2) TBDPS-Cl/imidazole, (3) H_2/Pd-C/sat. $NaHCO_3$ aq.; (b) (1) PCC, (2) $(EtO)_2P(O)CH_2COOEt$/NaH, (3) DIBALH, (4) Ac_2O/pyridine/DMAP; (c) (1) TBAF, (2) $(EtO)_2P(O)N_3$/DIAD/PPh_3, (3) LAH, (4) 4-phenylbenzoic acid or 4-bromobenzoic acid/HBTU/DIPEA; (d) $(CH_3CN)_2PdCl_2$; (e) (1) $K_2OsO_2(OH)_4$/$NaIO_4$, (2) H-His(Trt)-$N(OCH_3)CH_3$/$NaBH_3CN$; (f) (1) TFA, (2) DIBALH.

(HRMS) were obtained on a JMS-HX-110A (FAB), and Shimadzu LCMS-IT-TOF (ESI). Low-resolution mass spectra (LRMS) were obtained on a Shimadzu LCMS-2010EV (ESI). Optical rotations were determined with a HORIBA SEPA-300 polarimeter. Preparative HPLC was performed using a COSMOSIL 5C18-ARII column (20 × 250 mm) with a linear gradient of CH_3CN in 0.1% aqueous TFA at a flow rate of 5.0 mL/min on a HITACHI LaChrom system (OD, 254 nm). For analytical HPLC, unless otherwise noted, a COSMOSIL 5C18-ARII column (4.6 × 150 mm) was employed with a linear gradient of CH_3CN in 0.1% aqueous TFA at a flow rate of 0.9 mL/min on a HITACHI LaChrom system (OD, 254 nm). The purity of the test compounds was determined by analytical HPLC. All test compounds showed ⩾95% purity.

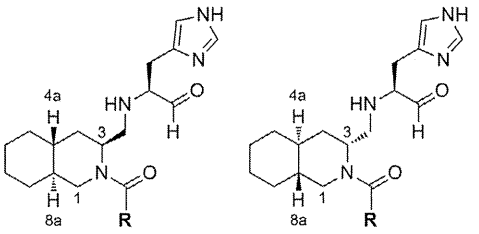
4.1.1. (1*S*/*R*,6*R*/*S*)-Ethyl 6-[2-(benzyloxy)ethyl]cyclohex-3-enecarboxylate 7

To a solution of 1,3-butadiene (20 wt% solution in hexane, 17 mL, 40 mmol) was added ester **6** (2.34 g, 10.0 mmol), heated at 250 °C for 60 h. After the reaction mixture was cooled to room temperature, water was added and the whole was extracted with AcOEt. The organic layer was washed with 1 M HCl and brine, dried over $MgSO_4$, filtered, and concentrated. The residue was purified by silica gel column chromatography (hexane/AcOEt = 30:1) to give **7** (1.87 g, 65%) as a yellow pale oil. ^1H NMR (400 MHz): δ = 7.36–7.31 (m, 4H), 7.29–7.26 (m, 1H), 5.64 (m, 2H), 4.51 (d, J = 11.6 Hz, 1H), 4.46 (d, J = 12.0 Hz, 1H), 4.14 (q, J = 7.2 Hz, 2H), 3.54–3.50 (m, 2H), 2.41–2.35 (m, 1H), 2.31–2.20 (m, 3H), 2.09–2.04 (m, 1H), 1.84–1.73 (m, 2H), 1.54–1.45 (m, 1H), 1.25 (t, J = 7.2 Hz, 3H); ^{13}C NMR (100 MHz): δ = 175.8, 138.5, 128.3, 127.6, 127.5, 125.7, 124.7, 72.8, 67.9, 60.2, 45.3, 33.7, 32.4, 29.9, 28.0, 14.2; HRMS (EI) calcd for $C_{18}H_{24}O_3$ $[M]^+$: 288.1725. Found: 288.1722.

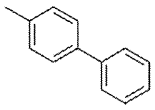
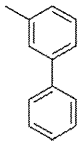
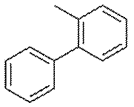
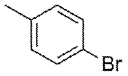
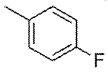
4.1.2. {(1*S*/*R*,6*R*/*S*)-6-[2-(Benzyloxy)ethyl]cyclohex-3-en-1-yl}methanol

To a suspension of $LiAlH_4$ (387 mg, 10.2 mmol) in ether (30 mL) was added **7** (1.47 g, 5.12 mmol) at 0 °C. After being stirred for 15 min at 0 °C, the reaction was quenched with H_2O. The mixture was warmed to room temperature and filtered through Celite and a silica gel layer, and the filtrate was concentrated. The residue was purified by silica gel column chromatography (hexane/AcOEt = 1:1) to give a title alcohol (1.25 g, quant.) as a colorless oil. ^1H NMR (400 MHz): δ = 7.37–7.32 (m, 4H), 7.30–7.26 (m, 1H), 5.65–5.57 (m, 2H), 4.51 (s, 2H), 3.66 (dd, J = 10.8, 6.0 Hz, 1H), 3.62–3.48 (m, 3H), 2.14–2.09 (m, 2H), 2.01–1.75 (m, 5H), 1.66–1.59 (m, 1H), 1.55–1.48 (m, 1H); ^{13}C NMR (100 MHz): δ = 138.3, 128.4, 127.7, 127.6, 125.8, 125.5, 73.1, 68.5, 65.0, 39.7, 32.9, 31.0, 29.5, 26.7; HRMS (EI) calcd for $C_{16}H_{22}O_2$ $[M]^+$: 246.1620. Found: 246.1618.

Table 1
Inhibitory activities of the decahydroisoquinolin derivatives



decahydroisoquinolin inhibitor

R	IC_{50}	
	(3*S*,4a*R*,8a*S*)	(3*R*,4a*S*,8a*R*)
	**40** 108 μM	**44** 240 μM
	**46** 135 μM	
	**47** 135 μM	
	**48** 68 μM	
	**41** 63 μM	45 175 μM
	**49** 57 μM	

4.1.3. ({(1*S*/*R*,6*R*/*S*)-6-[2-(Benzyloxy)ethyl]cyclohex-3-en-1-yl}methoxy)(*tert*-butyl)diphenylsilane 8

TBDPS-Cl (3.6 mL, 13.1 mmol) was added to a solution of the above alcohol (2.92 g, 11.9 mmol) and imidazole (1.21 g, 17.8 mmol) in CH_2Cl_2 (30 mL) and the mixture was stirred for 16 h. The reaction was quenched with saturated aqueous NH_4Cl, and the whole was extracted with AcOEt. The organic layer was washed with brine, dried over Na_2SO_4, filtered, and concentrated. The residue was purified by silica gel column chromatography (hexane/AcOEt = 20:1) to give 8 (5.76 g, quant.) as a colorless oil. ^1H NMR (400 MHz): δ = 7.67–7.65 (m, 4H), 7.43–7.30 (m, 10H), 7.28 (m, 1H), 5.63–5.54 (m, 2H), 4.49 (d, J = 12.0 Hz, 1H), 4.45 (d, J = 12.0 Hz, 1H), 3.68 (dd, J = 10.0, 5.2 Hz, 1H), 3.62 (dd, J = 9.8, 7.0 Hz, 1H,), 3.54–3.45 (m, 2H), 2.17–2.06 (m, 2H), 2.02–1.95, (m, 1H), 1.87–1.80 (m, 2H), 1.73–1.67 (m, 2H), 1.51–1.42 (m, 1H), 1.05 (s, 9H); ^{13}C NMR (100 MHz): δ = 138.6, 135.62, 135.61, 133.98, 133.95, 129.5, 128.3, 127.58, 127.56, 127.4, 125.8, 125.4, 72.9, 68.6, 65.9, 39.6, 32.9, 30.9, 29.1, 26.9, 26.7, 19.3; HRMS (FAB) calcd for $C_{32}H_{41}O_2Si$ $[M+H]^+$: 485.2876. Found: 485.2870.

4.1.4. 2-[(1*R*/*S*,6*S*/*R*)-6-{[(*tert*-Butyldiphenylsilyl)oxy]methyl}-cyclohexyl]ethanol

To a solution of **8** (3.40 g, 7.01 mmol) in CH_3OH/AcOEt/CH_2Cl_2 (10:10:1, 21 mL) $Pd(OH)_2$–C (610 mg) was added and stirred under a hydrogen gas atmosphere at room temperature for 12 h. The mixture was filtered through Celite and a silica gel layer, and the filtrate was dried over Na_2SO_4, filtered, and concentrated. The residue was purified by silica gel column chromatography (hexane/AcOEt = 3:1) to give a title alcohol (2.78 g, quant.) as a colorless oil. ^1H NMR (400 MHz): δ = 7.68–7.65 (m, 4H), 7.45–7.36 (m, 6H), 3.68–3.54 (m, 4H), 1.78–1.66 (m, 5H), 1.37–1.18 (m, 7H), 1.06 (s, 9H), 1.01–0.96 (m, 1H); ^{13}C NMR (100 MHz): δ = 135.69, 135.66, 133.92, 133.90, 129.55, 129.54, 127.60, 127.57, 66.6, 61.1, 44.5, 36.5, 35.5, 31.9, 30.0, 26.9, 26.1, 26.0, 19.3; HRMS (FAB) calcd for $C_{25}H_{37}O_2Si$ $[M+H]^+$: 397.2563. Found: 397.2569.

Table 2
Data collection and refinement statistics for the R188I SARS 3CL protease in complexes with compounds **40**, **41**, and **44**

PDB ID	4TWY In complex with **40**	4TWW In complex with **41**	4WY3 In complex with **44**
Space group	*C*121	*P*1	*C*121
Unit cell parameters			
Length *a*	107.83	54.89	108.11
Length *b*	82.128	59.52	81.82
Length *c*	53.271	68.40	53.24
Angle α	90	93.11	90
Angle β	104.98	102.82	104.69
Angle γ	90	107.30	90
Resolution	1.60	2.42	1.89
Observations			
Unique observations	57,490	31,213	49,270
Redundancy	4.2	1.75	4.1
Completeness	88.6	94.3	93.2
Mean I/σ (I)	2.18 (at 1.60 Å)	9.96 (at 2.42 Å)	2.49 (at 1.89 Å)
R merge	0.08	0.05	0.07
Refinement			
Resolution range	25.3–1.60	66.1–2.42	30.6–1.89
R_{cryst}	0.29	0.23	0.27
R_{free}	0.32	0.26	0.30
RMSZ from ideal			
Bond length (Å)	0.93	0.73	0.86
Bond angle (°)	0.96	0.86	0.90

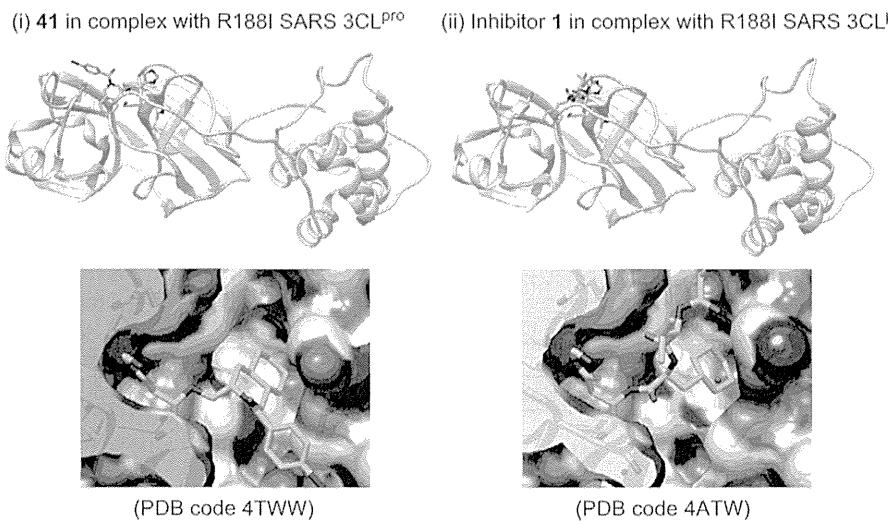


Figure 2. (i) X-ray structure of the inhibitor **41** in complex with R188I SARS 3CL^{pro} (PDB code 4TWW) and molecular graphics image around the P_1 and P_2 sites. (ii) X-ray structure of the inhibitor **1** in complex with R188I SARS 3CL^{pro} (Ref. 13; PDB code 4ATW) and molecular graphics image around the P_1 and P_2 sites.

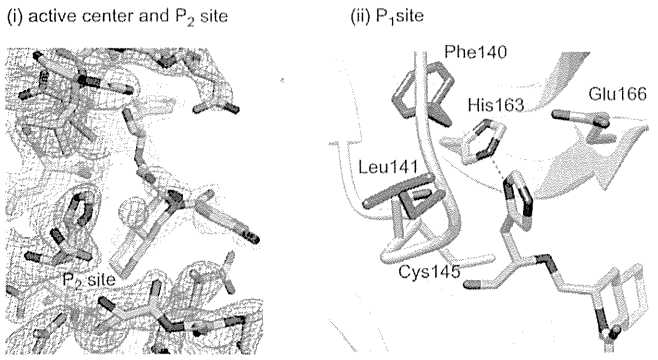


Figure 3. (i) Interactions of the inhibitor **41** with R188I SARS 3CL^{pro} at the active center and P_2 site. (ii) Interactions at the P_1site.

4.1.5. (*E*)-Ethyl 4-[(1*R*/*S*,2*S*/*R*)-2-{[(*tert*-butyldiphenylsilyl)oxy]methyl}cyclohex-1-yl]but-2-enoate 9

To a solution of PCC (3.45 g, 16.0 mmol) and Celite (3.5 g) in CH_2Cl_2 (20 mL), above alcohol (2.50 g, 6.30 mmol) was added at 0 °C. The temperature was gradually raised to room temperature. After being stirred for 6 h, the reaction mixture was filtered through a silica gel layer and the filtrate was concentrated. This compound was immediately used for the next step without purification. Triethylphosphonoacetate (1.5 mL, 7.7 mmol) was added to a suspension of NaH [60% in mineral oil (308 mg, 7.70 mmol)] in THF (10 mL) at −20 °C under an argon gas atmosphere and the mixture was stirred for 0.5 h. The oxidized product was added drop-wise to the reaction mixture and stirred for 1.5 h at −20 °C. The reaction was quenched with saturated aqueous NH_4Cl, and the whole was extracted with AcOEt. The organic layer was washed with brine, dried over Na_2SO_4, filtered, and concentrated. The residue was purified by silica gel column chromatography (hexane/AcOEt = 20:1) to give **9** (2.70 g, 92%, 2 steps) as a colorless oil. ^1H

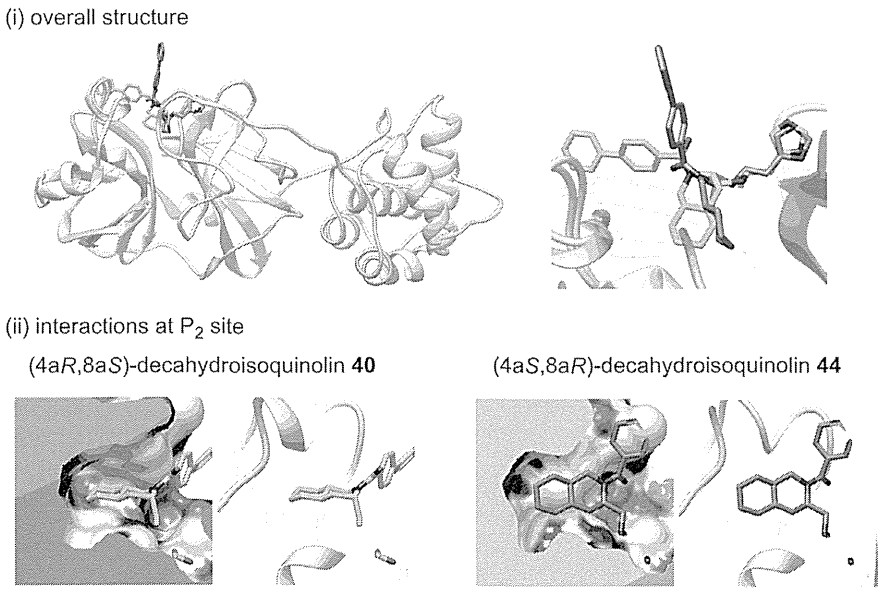


Figure 4. (i) X-ray structures of R188I SARS 3CL^{pro} in complex with (4a*R*/8a*S*)-*N*-4-phenylbenzoyl decahydroisoquinolin inhibitor **40** (PDB code 4TWY) and (4a*S*/8a*R*)-*N*-4-phenylbenzoyl decahydroisoquinolin inhibitor **44** (PDB code 4WY3). (ii) Interactions of **40** and **44** at the P_2 site.

NMR (400 MHz): δ = 7.67–7.64 (m, 4H) 7.45–7.36 (m, 6H), 6.91 (ddd, J = 15.4, 8.8, 6.4 Hz, 1H), 5.72 (d, J = 15.6 Hz, 1H), 4.18 (q, J = 7.1 Hz, 2H), 3.63–3.57 (m, 2H), 2.38–2.32 (m, 1H), 1.97 (td, J = 14.8, 8.1 Hz, 1H), 1.79–1.76 (m, 1H), 1.71–1.69 (m, 4H), 1.54–1.49 (m, 1H), 1.32–1.18 (m, 4H), 1.29 (t, J = 7.2 Hz, 3H), 1.05 (s, 9H), 1.03–0.97 (m, 1H); ^{13}C NMR (100 MHz): δ = 166.6, 148.2, 135.62, 135.61, 133.82, 133.80, 129.59, 129.55, 127.62, 127.59, 122.4, 66.2, 60.1, 43.9, 37.8, 36.4, 31.9, 30.0, 26.9, 26.1, 26.0, 19.3, 14.3; HRMS (FAB) calcd for $C_{29}H_{40}NaO_3Si$ $[M+Na]^+$: 487.2644. Found: 487.2651.

4.1.6. (*E*)-4-[(1*R*/*S*,2*S*/*R*)-2-{[(*tert*-Butyldiphenylsilyl)oxy]-methyl}cyclohexyl]but-2-en-1-ol

To a solution of **9** (1.92 g, 4.13 mmol) in CH_2Cl_2 (20 mL), DIBALH (1.0 mol/L solution in hexane, 12.4 mL, 12.4 mmol) was added at −78 °C. After being stirred for 15 min at the same temperature, the reaction was quenched with CH_3OH (5.0 mL). The mixture was warmed to room temperature, and filtered through Celite and a silica gel layer. The filtrate was dried over Na_2SO_4, filtered, and concentrated. The residue was purified by silica gel column chromatography (hexane/AcOEt = 1:1) to give a title alcohol (1.74 g, quant.) as a colorless oil. ^{1}H NMR (400 MHz): δ = 7.68–7.65 (m, 4H), 7.44–7.36 (m, 6H), 5.64–5.48 (m, 2H), 4.04 (d, J = 6.0 Hz, 2H), 3.66 (dd, J = 10.0, 2.8 Hz, 1H), 3.58 (dd, J = 9.8, 5.4 Hz, 1H), 2.23–2.17 (m, 1H), 1.87–1.79 (m, 2H), 1.72–1.69 (m, 3H), 1.43–1.32 (m, 1H), 1.30–1.18 (m, 4H), 1.05 (s, 9H), 1.01–0.94 (m, 1H); ^{13}C NMR (100 MHz): δ = 135.64, 135.63, 134.0, 131.6, 130.2, 129.52, 129.50, 127.58, 127.55, 66.3, 63.8, 43.9, 38.1, 36.2, 31.7, 30.0, 26.9, 26.2, 26.1, 19.4; HRMS (FAB) calcd for $C_{27}h_{38}nao_2si$ $[M+Na]^+$: 445.2539. Found: 445.2541.

4.1.7. (*E*)-4-[(1*R*/*S*,2*S*/*R*)-2-{[(*tert*-Butyldiphenylsilyl)oxy]-methyl}cyclohexyl]but-2-en-1-yl acetate 10

To a solution of above alcohol (1.74 g, 4.11 mmol) in CH_2Cl_2 (20 mL), pyridine (0.50 mL, 6.2 mmol), acetic anhydride (0.59 mL, 6.19 mmol), and DMAP (50 mg, 0.41 mmol) were added at 0 °C. The mixture was stirred at room temperature for 1 h. The reaction was quenched with saturated aqueous NH_4Cl. The mixture was extracted with AcOEt. The organic layer was washed with brine, dried over Na_2SO_4, filtered, and concentrated. The residue was purified by silica gel column chromatography (hexane/AcOEt = 30:1) to give **10** (1.81 g, 95%) as a colorless oil. ^{1}H NMR (400 MHz): δ = 7.67–7.64 (m, 4H), 7.44–7.36 (m, 6H), 5.71–5.64 (m, 1H), 5.49–5.42 (m, 1H), 4.47 (d, J = 6.4 Hz, 2H), 3.65 (dd, J = 9.8, 3.0 Hz, 1H), 3.57 (dd, J = 10.0, 4.8 Hz, 1H), 2.23–2.18 (m, 1H), 2.05 (s, 3H), 1.87–1.79 (m, 2H), 1.71–1.68 (m, 3H), 1.43–1.35 (m, 1H), 1.30–1.18 (m, 4H), 1.05 (s, 9H), 1.00–0.94 (m, 1H); ^{13}C NMR (100 MHz): δ = 170.9, 135.6, 134.8, 133.94, 133.93, 129.5, 127.6, 125.0, 66.3, 65.3, 43.8, 38.0, 36.3, 31.7, 30.0, 26.9, 26.2, 26.1, 21.0, 19.3; HRMS (FAB) calcd for $C_{29}H_{40}NaO_3Si$ $[M+Na]^+$: 487.2644. Found: 487.2642.

4.1.8. (*E*)-4-[(1*R*/*S*,2*S*/*R*)-2-(Hydroxymethyl)cyclohexyl]but-2-en-1-yl acetate

To a solution of **10** (1.81 g, 3.89 mmol) in THF (20 mL), TBAF [1.0 M solution in THF (7.8 mL, 7.8 mmol)] was added at room temperature. After the mixture was stirred for 12 h, the reaction was quenched with saturated aqueous NH_4Cl and the whole was extracted with AcOEt. The organic layer was washed with brine, dried over Na_2SO_4, filtered, and concentrated. The residue was purified by silica gel column chromatography (hexane/AcOEt = 6:1) to give a title alcohol (1.03 g, quant.) as a colorless oil. ^{1}H NMR (400 MHz): δ = 5.80–5.72 (m, 1H), 5.60–5.53 (m, 1H), 4.51 (d, J = 6.4 Hz, 2H), 3.69 (dd, J = 10.8, 3.2 Hz, 1H), 3.59 (dd, J = 10.8, 5.6 Hz, 1H), 2.33–2.27 (m, 1H), 2.06 (s, 3H), 2.02–1.90 (m, 1H), 1.81–1.79 (m, 1H), 1.74–1.67 (m, 3H), 1.37–1.11 (m, 5H), 1.05–0.95 (m, 1H); ^{13}C NMR (100 MHz): δ = 170.9, 134.5, 125.3, 65.7, 65.2, 43.8, 38.0, 36.4, 31.7, 29.5, 26.0, 25.8, 21.0; HRMS (FAB) calcd for $C_{13}H_{22}NaO_3$ $[M+Na]^+$: 249.1467. Found: 249.1460.

4.1.9. *N*-({(1*S*/*R*,2*R*/*S*)-2-[(*E*)-4-Hydroxybut-2-en-1-yl]cyclohexyl}methyl)-[1,1′-biphenyl]-4-carboxamide 12

DPPA (2.4 mL, 11 mmol) was added drop-wise to a solution of above alcohol (1.03 g, 4.56 mmol), triphenylphosphine (2.80 g, 10.8 mmol), and DEAD (40% solution in toluene, 4.2 mL, 10.8 mmol) in THF (10 mL) at 0 °C. The mixture was stirred for 16 h at the same temperature, and then the reaction mixture was concentrated. The residue was roughly purified by silica gel column chromatography (hexane/AcOEt = 30:1) to give **11**. ^{1}H NMR (400 MHz): δ = 5.77–5.69 (m, 1H), 5.62–5.54 (m, 1H), 4.52 (d, J = 6.0 Hz, 2H), 3.40 (dd, J = 12.0, 3.2 Hz, 1H), 3.25 (dd, J = 12.2, 6.2 Hz, 1H), 2.29–2.24 (m, 1H), 2.06 (s, 3H), 2.00–1.91 (m, 1H), 1.80–1.65 (m, 4H), 1.34–1.29 (m, 2H), 1.27–1.11 (m, 3H), 1.05–0.95 (m, 1H).

The crude **11** was dissolved in ether (10 mL) and added to a suspension of $LiAlH_4$ (1.04 g, 27.4 mmol) in ether (10 mL) at 0 °C. The reaction was quenched with CH_3OH and concentrated. The mixture was stirred for 6 h under reflux. The reaction mixture cooled to room temperature and then quenched with CH_3OH and concentrated to give a corresponding amine derivative. ^{1}H NMR (400 MHz): δ = 5.63–5.48 (m, 2H), 3.94–3.92 (m, 2H), 2.81 (dd, J = 12.6, 3.0 Hz, 1H), 2.43 (dd, J = 12.8, 7.6 Hz, 1H), 2.20–2.15 (m, 1H), 1.97–1.86 (m, 1H), 1.79–1.75 (m, 1H), 1.69–1.60 (m, 3H), 1.24–1.09 (m, 5H), 1.05–0.96 (m, 1H).

The residue was used in the next step without purification. The crude product in CH_2Cl_2 (10 mL) was added to a solution of HBTU (4.32 g, 11.4 mmol), DIPEA (2.4 mL, 14 mmol), and 4-biphenyl carboxylic acid (903 mg, 4.56 mmol) in CH_2Cl_2 (10 mL) at 0 °C. The mixture was stirred for 3 h at room temperature. The reaction was quenched with saturated aqueous NH_4Cl and extracted with CH_2Cl_2. The organic layer was washed with brine and dried over Na_2SO_4, filtered, and concentrated. The residue was purified by silica gel column chromatography (hexane/AcOEt = 1:1) to afford **12** (1.04 g, 63%, 3 steps) as a colorless oil. ^{1}H NMR (400 MHz): δ = 7.84–7.82 (m, 2H), 7.67–7.59 (m, 4H), 7.48–7.44 (m, 2H), 7.41–7.37 (m, 1H), 6.28 (br s, 1H), 5.79–5.67 (m, 2H), 4.10 (d, J = 4.4 Hz, 2H), 3.78 (ddd, J = 13.6, 6.0, 3.6 Hz, 1H), 3.20 (ddd, J = 13.7, 8.1, 5.9 Hz, 1H), 2.32–2.27 (m, 1H), 2.21–2.12 (m, 1H), 1.87–1.84 (m, 1H), 1.74–1.72 (m, 3H), 1.52–1.41 (m, 1H), 1.32–1.04 (m, 5H); ^{13}C NMR (100 MHz): δ = 167.2, 144.2, 140.0, 133.3, 131.2, 130.5, 128.9, 128.0, 127.3, 127.23, 127.17, 63.8, 43.3, 41.1, 39.6, 36.5, 31.9, 30.6, 26.0, 25.7; HRMS (EI) calcd for $C_{24}H_{29}NO_2$ $[M]^+$:363.2198. Found: 363.2207.

4.1.10. 4-Bromo-*N*-({(1*S*/*R*,2*R*/*S*)-2-[(*E*)-4-hydroxybut-2-en-1-yl]cyclohexyl}methyl)benzamide 13

A title compound was similarly prepared from **10** as above. Colorless oil; yield 50% (3 steps): ^{1}H NMR (400 MHz): δ = 7.64–7.61 (m, 2H), 7.58–7.56 (m, 2H), 6.16 (m, 1H), 5.78–5.66 (m, 2H), 4.10 (d, J = 4.8 Hz, 2H), 3.76 (ddd, J = 13.4, 5.8, 3.8 Hz, 1H), 3.16 (ddd, J = 13.7, 8.1, 5.9 Hz, 1H), 2.29–2.25 (m, 1H), 2.18–2.11 (m, 1H), 1.84–1.80 (m, 1H), 1.73–1.71 (m, 3H), 1.50–1.41 (m, 1H), 1.28–0.96 (m, 5H); ^{13}C NMR (100 MHz): δ = 166.5, 133.5, 131.8, 131.2, 130.4, 128.5, 126.0, 63.7, 43.3, 41.0, 39.6, 36.4, 31.9, 30.5, 26.0, 25.7; HRMS (EI) calcd for $C_{18}H_{24}BrNO_2$ $[M]^+$ 365.0990. Found 365.0996.

4.1.11. (1,1′-Biphenyl)-4-yl{(3*S*/*R*,4a*R*/*S*,8a*S*/*R*)-3-vinyloctahydroisoquinolin-2(1*H*)-yl}methanone 14

To a solution of **12** (120 mg, 0.331 mmol) in dry CH_2Cl_2 (1 mL), $(CH_3CN)_2PdCl_2$ (15 mg, 0.056 mmol) was added at 0 °C under an argon gas atmosphere, and the mixture was stirred at the same

temperature for 4 h. The reaction mixture was filtered and concentrated. The residue was purified by silica gel column chromatography (hexane/AcOEt = 10:1) to give **14** (100 mg, 88%) as a colorless oil. ^{1}H NMR (400 MHz): δ = 7.64–7.58 (m, 4H), 7.49–7.43 (m, 4H), 7.38–7.35 (m, 1H), 5.87 (ddd, J = 17.5, 10.7, 3.7 Hz, 0.4H), 5.78 (ddd, J = 17.5, 10.7, 3.5 Hz, 0.6H), 5.55 (br s, 0.4H), 5.31–5.28 (m, 1H), 5.23–5.16 (m, 1H), 4.54 (br s, 0.6H), 4.49 (dd, J = 13.2, 4.0 Hz, 0.6H), 3.49 (dd, J = 13.0, 3.8 Hz, 0.4H), 2.86 (dd, J = 13.2, 11.6 Hz, 0.4H), 2.61 (dd, J = 12.8, 11.6 Hz, 0.6H), 1.84–1.52 (m, 5H), 1.47–1.18 (m, 5H), 1.15–1.13 (m, 0.4H), 1.03–0.98 (m, 1H), 0.90–0.84 (m, 0.6H); ^{13}C NMR (100 MHz): δ = 171.1, 170.4, 142.3, 142.2, 140.3, 137.1, 136.7, 135.4, 128.8, 127.69, 127.66, 127.4, 127.1, 126.8, 116.6, 116.1, 57.2, 50.8, 49.7, 43.5, 42.8, 41.9, 37.5, 36.8, 35.9, 32.9, 29.9, 29.7, 26.2, 26.1, 25.8, 25.7; HRMS (EI) calcd for $C_{24}H_{27}NO$ [M]$^+$: 345.2093. Found: 345.2090.

4.1.12. (4-Bromophenyl)((3*S*,4a*R*/*S*,8a*S*/*R*)-3-vinyloctahydroisoquinolin-2(1*H*)-yl)methanone 15

A title compound was similarly prepared as above. Colorless oil; yield 57%: ^{1}H NMR (400 MHz): δ = 7.56–7.50 (m, 2H), 7.29–7.27 (m, 2H), 5.84 (ddd, J = 17.4, 10.6, 3.8 Hz, 0.4H), 5.74 (ddd, J = 17.5, 10.7, 3.5 Hz, 0.6H), 5.49 (br s, 0.4H), 5.29–5.26 (m, 1H), 5.19–5.10 (m, 1H), 4.44 (dd, J = 13.4, 3.8 Hz, 0.6H), 4.39 (s, 0.6H), 3.33 (dd, J = 13.2, 3.6 Hz, 0.4H), 2.82 (dd, J = 13.0, 11.8 Hz, 0.4H), 2.57 (dd, J = 13.0, 11.4 Hz, 0.6H), 1.83–1.49 (m, 5H), 1.43–1.19 (m, 5H), 1.13–1.04 (m, 0.4H), 0.99–0.96 (m, 1H), 0.88–0.83 (m, 0.6H); ^{13}C NMR (100 MHz): δ = 170.2, 169.6, 136.9, 136.5, 135.4, 131.7, 131.6, 128.6, 128.0, 123.7, 123.6, 116.7, 116.2, 57.2, 50.8, 49.6, 43.5, 42.8, 41.8, 37.5, 36.7, 35.9, 32.8, 29.9, 29.6, 26.1, 26.0, 25.7, 25.6; HRMS (EI) Calcd for $C_{18}H_{22}BrNO$ [M]$^+$: 347.0885. Found: 347.0879.

4.1.13. (*S*)-2-({[(3*S*,4a*R*,8a*S*)-2-[(1,1′-Biphenyl)-4-carbonyl]-decahydroisoquinolin-3-yl]methyl}amino)-*N*-methoxy-*N*-methyl-3-(1-trityl-1*H*-imidazol-4-yl)propanamide 18

To a solution of $K_2OsO_2(OH)_4$ (3.1 mg, 0.0083 mmol) and *N*-methylmorpholine *N*-oxide (389 mg, 3.32 mmol), **14** (286 mg, 0.829 mmol) was added in THF/H_2O (3:1, 10 mL). After being stirred for 12 h, $NaIO_4$ (710 mg, 3.32 mmol) was added to the mixture. The resultant mixture was stirred for 30 min. The reaction was quenched with H_2O, and the whole was extracted with AcOEt. The organic layer was washed with brine, dried over $MgSO_4$, filtered, and concentrated. The residue was roughly purified by silica gel column chromatography (hexane/AcOEt = 3:1) to give **16**. ^{1}H NMR (400 MHz): δ = 9.69 (s, 0.75H), 9.65 (s, 0.25H), 7.69–7.54 (m, 5H), 7.49–7.37 (m, 4H), 5.50 (d, J = 6.4 Hz, 0.75H), 4.62–4.59 (m, 0.25H), 4.44 (d, J = 5.6 Hz, 0.25H), 3.69–3.65 (m, 0.75H), 2.81 (dd, J = 13.2, 11.6 Hz, 0.75H), 2.40 (t, J = 12.6 Hz, 0.25H), 2.33 (d, J = 13.6 Hz, 0.75H), 2.15 (dd, J = 13.6 Hz, 0.25H), 1.74–1.69 (m, 3H), 1.59–1.50 (m, 1H), 1.44–1.41 (m, 1H), 1.25–1.11 (m, 3H), 1.08–0.96 (m, 2H), 0.92–0.76 (m, 1H).

The product was used without further purification. To a solution of **16** and H-His(Trt)-N(OCH_3)CH_3 (410 mg, 0.930 mmol) in CH_2Cl_2 (1 mL), AcOH (0.05 mL, 0.8 mmol) was added. The mixture was stirred at room temperature for 2 h and then $NaBH_3CN$ (181 mg, 2.88 mmol) was added. The resultant mixture was stirred for 30 min. The reaction was quenched with 1 M HCl and the whole was extracted with AcOEt. The organic layer was washed with saturated aqueous $NaHCO_3$ and brine, dried over Na_2SO_4, filtered, and concentrated. The residue was purified by flash column chromatography ($CHCl_3$/CH_3OH = 25:1) to give **18** and **20**.

*Compound **18**:* [80 mg, 13% (50% max.), 3 steps] as a colorless oil. $[\alpha]_D^{28}$ −20 (*c* 0.48, $CHCl_3$); ^{1}H NMR (400 MHz): δ = 7.59–7.53 (m, 4H), 7.47–7.41 (m, 4H), 7.37–7.29 (m, 11H), 7.13–7.09 (m, 6H), 6.62 (m, 0.6H), 6.56 (m, 0.4H), 4.94 (br s, 0.6H), 4.41 (dd, J = 13.0, 3.0 Hz, 0.4H), 4.12–4.11 (m, 0.4H), 3.93 (m, 1H), 3.69 (s, 1.8H), 3.50 (s, 1.2H), 3.44–3.41 (m, 0.6H), 3.14 (s, 1.8H), 3.08 (s, 1.2H), 2.93–2.84 (m, 2.4H), 2.76–2.66 (m, 2H), 2.46 (t, J = 12.2 Hz, 0.6H), 1.80–1.70 (m, 3H), 1.61–1.54 (m, 1H), 1.43–1.17 (m, 6H), 1.08–0.85 (m, 2H); ^{13}C NMR (100 MHz): δ = 175.4, 175.2, 171.3, 170.5, 142.44, 142.38, 141.88, 141.86, 140.42, 140.35, 138.2, 138.1, 137.6, 137.2, 135.9, 135.7, 129.72, 129.66, 129.3, 128.8, 128.7, 127.91, 127.87, 127.54, 127.46, 127.4, 127.2, 127.1, 127.04, 126.99, 119.3, 115.6, 77.2, 75.03, 75.02, 61.6, 61.5, 57.8, 57.4, 55.5, 49.5, 48.3, 47.1, 46.6, 43.1, 42.6, 42.1, 36.4, 36.2, 34.4, 33.0, 32.9, 32.6, 32.2, 32.0, 29.9, 29.7, 26.14, 26.05, 25.8, 25.7; HRMS (EI) calcd for $C_{50}H_{53}N_5O_3$ [M]$^+$: 771.4148. Found: 771.4141.

*Compound **20**:* [75 mg, 12% (50% max.), 3 steps] as a colorless oil. $[\alpha]_D^{28}$ +32 (*c* 2.3, $CHCl_3$); ^{1}H NMR (400 MHz): δ = 7.58–7.24 (m, 19H), 7.13–7.07 (m, 6H), 6.58 (m, 0.4H), 6.55 (m, 0.6H), 5.02–4.97 (m, 0.4H), 4.46 (dd, J = 13.2, 3.6 Hz, 0.6H), 4.13 (br s, 0.4H), 3.95 (m, 1H), 3.65 (s, 1.2H), 3.62–3.58 (m, 0.6H), 3.50 (s, 1.8H), 3.44 (dd, J = 13.4, 3.4 Hz, 0.4H), 3.14 (s, 1.2H), 3.11 (s, 1.8H), 3.01–2.94 (m, 1H), 2.89–2.81 (m, 2H), 2.65 (dd, J = 11.8, 6.6 Hz, 0.4H), 2.52 (dd, J = 12.0, 6.8 Hz, 0.6H), 2.50–2.44 (m, 0.6H), 2.26–2.24 (br s, 1H), 1.71–1.69 (m, 3H), 1.60–1.52 (m, 2H), 1.45–1.16 (m, 5H), 1.07–0.83 (m, 2H); ^{13}C NMR (100 MHz): δ = 175.6, 175.3, 171.1, 170.8, 142.44, 142.37, 141.9, 141.8, 140.41, 140.39, 138.12, 138.08, 137.5, 137.3, 135.7, 129.72, 129.66, 128.73, 128.68, 127.9, 127.5, 127.4, 127.1, 127.05, 127.03, 126.95, 119.5, 119.3, 77.2, 75.0, 61.6, 61.5, 57.7, 57.5, 55.4, 49.3, 48.4, 47.4, 47.2, 43.0, 42.8, 42.0, 36.7, 36.5, 34.6, 33.5, 33.00, 32.96, 32.3, 32.1, 29.9, 29.7, 29.6, 26.2, 26.0, 25.8, 25.7; HRMS (EI) calcd for $C_{50}H_{53}N_5O_3$ [M]$^+$: 771.4148. Found: 771.4154.

4.1.14. (*S*)-2-({[(3*S*,4a*R*,8a*S*)-2-(4-Bromobenzoyl)decahydroisoquinolin-3-yl]methyl}amino)-*N*-methoxy-*N*-methyl-3-(1-trityl-1*H*-imidazol-4-yl)propanamide 19

Compound **19** was similarly synthesized as **18**. Colorless oil; yield 11% (50% max., 3 steps): $[\alpha]_D^{28}$ −31 (*c* 0.83, $CHCl_3$); ^{1}H NMR (400 MHz): δ = 7.47 (d, J = 8.4 Hz, 1.2H), 7.44 (d, J = 8.4 Hz, 0.8H), 7.34–7.31 (m, 10.8H), 7.22 (d, J = 8.4 Hz, 1.2H), 7.12–7.11 (m, 6H), 6.60 (br s, 0.6H), 6.55 (br s, 0.4H), 4.87 (m, 0.6H), 4.37 (dd, J = 13.2, 3.6 Hz, 0.4H), 4.10 (br s, 0.6H), 3.89 (br s, 0.4H), 3.78 (m, 0.6H), 3.64 (s, 1.8H), 3.51 (s, 1.2H), 3.24 (dd, J = 13.2, 3.6 Hz, 0.6H), 3.13 (s, 1.8H), 3.11 (s, 1.2H), 2.91–2.80 (m, 2.4H), 2.73–2.62 (m, 2H), 2.47–2.41 (m, 0.4H), 1.76–1.65 (m, 3.4H), 1.60–1.54 (m, 1.6H), 1.36–1.25 (m, 5H), 1.00–0.82 (m, 2H); ^{13}C NMR (100 MHz): δ = 175.5, 175.1, 170.4, 169.7, 142.5, 142.4, 138.3, 138.1, 137.7, 137.2, 135.9, 135.8, 131.52, 131.49, 129.75, 129.72, 128.7, 128.4, 127.94, 127.91, 123.24, 123.21, 119.26, 119.25, 77.2, 75.1, 61.6, 61.5, 57.8, 57.5, 55.6, 49.3, 48.4, 47.1, 46.6, 43.1, 42.6, 42.0, 36.4, 36.2, 34.5, 33.0, 32.9, 32.7, 32.3, 32.0, 29.9, 29.7, 26.1, 26.0, 25.8, 25.7; HRMS (EI) calcd for $C_{44}H_{48}BrN_5O_3$ [M]$^+$: 773.2941. Found: 773.2948.

4.1.15. (*S*)-2-({[(3*R*,4a*S*,8a*R*)-2-(4-Bromobenzoyl)decahydroisoquinolin-3-yl]methyl}amino)-*N*-methoxy-*N*-methyl-3-(1-trityl-1*H*-imidazol-4-yl)propanamide 21

Compound **21** was similarly synthesized as **20**. Colorless oil; yield 11% (50% max., 3 steps): $[\alpha]_D^{28}$ +4.5 (*c* 0.42, $CHCl_3$); ^{1}H NMR (400 MHz): δ = 7.47–7.43 (m, 2H), 7.37–7.29 (m, 12H), 7.12–7.10 (m, 6H), 6.55 (m, 1H), 4.98–4.95 (m, 0.4H), 4.40 (dd, J = 13.2, 3.6 Hz, 0.6H), 4.10 (br s, 0.4H), 3.90 (br s, 0.6H), 3.84–3.81 (m, 0.6H), 3.64–3.58 (m, 0.4H), 3.63 (s, 1.8H), 3.54 (s, 1.2H), 3.28 (dd, J = 13.2, 3.6 Hz, 0.4H), 3.13 (s, 1.8H), 3.11 (s, 1.2H), 2.98–2.91 (m, 1H), 2.86–2.74 (m, 2.6H), 2.60 (dd, J = 11.6, 6.0 Hz, 0.6H), 2.47–2.41 (m, 1.4H), 1.72–1.65 (m, 3H), 1.59–1.47 (m, 2H), 1.43–1.12 (m, 5H), 1.04–0.79 (m, 2H); ^{13}C NMR (100 MHz): δ = 175.6, 175.2, 170.3, 170.0, 142.42, 142.37, 138.2, 138.1, 137.35, 137.25, 135.70, 135.66, 131.47, 131.45, 129.73, 129.70, 128.9, 128.7, 127.93, 127.91, 123.32, 123.26, 119.5, 119.3, 77.2, 75.1, 75.0, 61.5, 57.5,

57.4, 55.5, 49.2, 48.4, 47.4, 47.2, 42.9, 42.8, 42.0, 36.6, 36.5, 34.7, 33.6, 33.0, 32.9, 32.3, 32.0, 29.8, 29.6, 26.1, 26.0, 25.8, 25.6; HRMS (EI) calcd for $C_{44}H_{48}BrN_5O_3$ $[M]^+$: 773.2941. Found: 773.2944.

4.1.16. (*S*)-2-[({(3*S*,4a*R*,8a*S*)-2-[(1,1′-Biphenyl)-4-carbonyl]decahydroisoquinolin-3-yl}methyl)amino]-3-(1*H*-imidazol-4-yl)-*N*-methoxy-*N*-methylpropanamide

TFA/CH_2Cl_2/TIS/H_2O (10:10:1.0:1.0, 5.5 mL) was added to **18** (40 mg, 0.052 mmol). The mixture was stirred at room temperature for 4 h. The mixture was concentrated under reduced pressure. The residue was diluted with AcOEt and basified by saturated aqueous $NaHCO_3$. The whole was extracted with AcOEt and the organic layer was washed with brine, dried over Na_2SO_4, filtered, and concentrated. The residue was purified by silica gel column chromatography ($CHCl_3$/CH_3OH = 10:1) to give the de-tritylated product (25 mg, 90%) as a yellowish oil. $[\alpha]_D^{28}$ −33 (*c* 0.51, $CHCl_3$); ^1H NMR (400 MHz): δ = 7.68–7.36 (m, 10H), 6.84 (s, 0.6H), 6.82 (s, 0.4H), 5.03–5.01 (m, 0.4H), 4.31–4.27 (m, 0.6H), 4.15 (br s, 0.6H), 3.86 (br s, 0.4H), 3.73 (s, 1.2H), 3.66 (s, 1.8H), 3.54–3.51 (m, 1H), 3.25 (s, 1.2H), 3.19 (s, 1.8H), 3.00–2.86 (m, 1H), 2.75–2.62 (m, 2H), 2.52–2.44 (m, 2H), 1.77–1.68 (m, 3.4H), 1.62–1.59 (m, 1.6H), 1.49–1.23 (m, 5H), 1.17–1.11 (m, 0.4H), 1.07–0.99 (m, 1H), 0.89–0.85 (m, 0.6H); ^{13}C NMR (100 MHz): δ = 174.4, 171.0, 143.1, 142.4, 140.2, 140.1, 135.8, 135.3, 135.2, 134.8, 128.84, 128.83, 128.2, 127.8, 127.7, 127.5, 127.20, 127.18, 127.14, 127.08, 77.2, 61.7, 59.8, 58.4, 55.7, 49.5, 49.4, 48.6, 48.1, 43.5, 42.6, 42.0, 36.7, 34.3, 34.1, 33.0, 32.9, 32.2, 30.0, 29.6, 26.2, 26.0, 25.8, 25.6; HRMS (EI) calcd for $C_{31}H_{39}N_5O_3$ $[M]^+$: 529.3053. Found: 529.3057.

Compounds **19**, **20**, and **21** were similarly treated with TFA/CH_2Cl_2/TIS/H_2O (10:10:1.0:1.0, 5.5 mL) as above to yield the corresponding de-tritylated products.

4.1.17. From 19: (*S*)-2-({[(3*S*,4a*R*,8a*S*)-2-(4-bromobenzoyl)-decahydroisoquinolin-3-yl]methyl}amino)-3-(1*H*-imidazol-4-yl)-*N*-methoxy-*N*-methylpropanamide

Yellowish oil; yield, 70%: $[\alpha]_D^{28}$ −33.9 (*c* 0.415, $CHCl_3$); ^1H NMR (400 MHz): δ = 7.66 (s, 0.6H), 7.56–7.53 (m, 2H), 7.38 (s, 0.4H), 7.32 (d, *J* = 8.4 Hz, 1.2H), 7.19 (d, *J* = 8.4 Hz, 0.8H), 6.83 (s, 0.6H), 6.81 (s, 0.4H), 4.97–4.95 (m, 0.4H), 4.26–4.22 (m, 0.6H), 4.00–3.98 (m, 0.6H), 3.85–3.84 (m, 0.4H), 3.72 (s, 1.2H), 3.66 (s, 1.8H), 3.56–3.53 (m, 0.6H), 3.35 (dd, *J* = 13.4, 3.8 Hz, 0.4H), 3.24 (s, 1.2H), 3.20 (s, 1.8H), 2.99–2.83 (m, 2H), 2.71–2.60 (m, 2H), 2.54–2.41 (m, 2H), 1.77–1.58 (m, 4H), 1.51–1.33 (m, 1H), 1.30–1.17 (m, 5H), 1.05–0.80 (m, 2H); ^{13}C NMR (100 MHz): δ = 174.3, 173.1, 170.2, 135.8, 135.4, 135.3, 134.7, 131.9, 131.7, 129.3, 128.3, 124.2, 123.7, 77.2, 61.7, 59.7, 58.4, 55.8, 49.6, 49.4, 48.5, 48.0, 43.5, 42.6, 42.0, 36.6, 34.2, 34.0, 33.0, 32.8, 32.6, 29.9, 29.6, 26.1, 26.0, 25.8, 25.6; HRMS (EI) calcd for $C_{25}H_{34}BrN_5O_3$ $[M]^+$: 531.1845. Found: 531.1839.

4.1.18. From 20: (*S*)-2-[({(3*R*,4a*S*,8a*R*)-2-[(1,1′-biphenyl)-4-carbonyl]decahydroisoquinolin-3-yl}methyl)amino]-*N*-methoxy-*N*-methyl-3-(1*H*-imidazol-4-yl)propanamide

Yellowish oil; yield, quantitative: $[\alpha]_D^{28}$ −41 (*c* 0.45, $CHCl_3$); ^1H NMR (400 MHz): δ = 7.65–7.59 (m, 4H), 7.54 (s, 1H), 7.49–7.44 (m, 4H), 7.39–7.36 (m, 1H), 6.78 (m, 1H), 5.21–5.20 (m, 0.75H), 4.52–4.49 (m, 0.25H), 4.12 (m, 0.25H), 3.90–3.88 (m, 0.75H), 3.67 (s, 2.25H), 3.67–3.65 (m, 0.75H), 3.56 (s, 0.75H), 3.56–3.49 (m, 0.75H), 3.25 (s, 2.25H), 3.25–3.21 (m, 0.25H), 3.21 (s, 0.75H), 3.11–3.05 (m, 0.25H), 2.98–2.95 (m, 0.75H), 2.89–2.83 (m, 0.75H), 2.63–2.52 (m, 1.5H), 2.37 (dd, *J* = 12.0, 4.4 Hz, 1H), 2.29 (m, 1H), 1.72 (br s, 2H), 1.62–1.41 (m, 4H), 1.30–1.22 (m, 4H), 1.19–1.06 (m, 0.75H), 1.00–0.85 (m, 1.25H); ^{13}C NMR (100 MHz): δ = 174.9, 171.6, 171.0, 142.4, 140.2, 135.6, 135.4, 135.2, 134.4, 128.9, 128.8, 127.7, 127.4, 127.23, 127.16, 127.1, 127.0, 77.2, 61.7, 58.5, 55.5, 49.4, 49.1, 47.5, 42.8, 42.3, 36.9, 36.8, 35.2, 34.3, 33.1, 32.9, 32.3, 29.9, 29.65, 29.56, 29.2, 26.2, 26.0, 25.8, 25.6; HRMS (EI) calcd for $C_{31}H_{39}N_5O_3$ $[M]^+$: 529.3053. Found: 529.3060.

4.1.19. From 21: (*S*)-2-({[(3*R*,4a*S*,8a*R*)-2-(4-bromobenzoyl) decahydroisoquinolin-3-yl]methyl}amino)-*N*-methoxy-*N*-methyl-3-(1*H*-imidazol-4-yl)propanamide

Yellowish oil; yield, 65%: $[\alpha]_D^{28}$ −27.7 (*c* 0.96, $CHCl_3$); ^1H NMR (400 MHz): δ = 7.57–7.47 (m, 3H), 7.30–7.27 (m, 2H), 6.79 (s, 0.25H), 6.78 (s, 0.75H), 5.17–5.14 (m, 0.75H), 4.45 (dd, *J* = 13.4, 3.4 Hz, 0.25H), 3.94 (br s, 0.25H), 3.87–3.86 (m, 0.75H), 3.67 (s, 2.25H), 3.59 (s, 0.75H), 3.39 (dd, *J* = 13.6, 3.2 Hz, 0.75H), 3.25 (s, 2.25H), 3.21 (s, 0.75H), 3.19–3.16 (m, 0.75H), 3.07–3.01 (m, 0.25H), 2.98–2.89 (m, 1H), 2.82 (dd, *J* = 13.4, 11.8 Hz, 0.75H), 2.70–2.48 (m, 1.5H), 2.36 (dd, *J* = 12.2, 4.6 Hz, 0.75H), 2.30 (dd, *J* = 11.8, 5.8 Hz, 0.25H), 1.82–1.61 (m, 5H), 1.48–1.28 (m, 5H), 1.09–1.04 (m, 0.75H), 0.98–0.87 (m, 1.25H); ^{13}C NMR (100 MHz): δ = 174.9, 170.7, 170.2, 135.6, 135.31, 135.26, 134.5, 131.8, 131.6, 128.7, 128.4, 123.8, 123.5, 77.2, 61.7, 58.4, 58.0, 55.4, 49.5, 49.1, 47.5, 47.4, 42.8, 42.7, 42.2, 36.8, 36.7, 35.1, 34.2, 33.0, 32.9, 32.3, 29.8, 29.5, 29.2, 26.1, 26.0, 25.8, 25.6; HRMS (EI) Calcd. For $C_{25}H_{34}BrN_5O_3$ $[M]^+$: 531.1845. Found: 531.1839.

4.1.20. (*S*)-2-[({(3*S*,4a*R*,8a*S*)-2-[(1,1′-Biphenyl)-4-carbonyl]decahydroisoquinolin-3-yl}methyl)amino]-3-(1*H*-imidazol-4-yl)-propanal 22

To a solution of above de-tritylated product of **18** (33 mg, 0.61 mmol) in CH_2Cl_2 (1 mL), DIBALH (1.0 mol/L solution in hexane, 1.2 mL, 1.2 mmol) was added drop-wise at −78 °C. The reaction mixture was stirred for 5 min. The reaction was quenched with CH_3OH and concentrated. The residue was dissolved in CH_3OH and filtered through a silica gel layer. The filtrate was concentrated. The residue was purified by HPLC to give **22** (10.5 mg, 28%) as a colorless oil. $[\alpha]_D^{28}$ −3.2 (*c* 0.48, CH_3OH); ^1H NMR (500 MHz, CD_3OD, referenced to residual CH_3OH): δ = 8.80 (br s, 1H), 7.75–7.73 (m, 2H), 7.67–7.65 (m, 2H), 7.58 (d, *J* = 8.4 Hz, 2H), 7.50 (br s, 1H), 7.48–7.45 (m, 2.5H), 7.40–7.36 (m, 1.5H), 5.13 (m, 1H), 4.82 (dd, *J* = 8.4, 3.2 Hz, 1H), 3.88–3.79 (m, 2H), 3.63–3.59 (m, 1H), 3.43–3.40 (m, 1H), 3.34 (s, 1H), 2.97 (t, *J* = 12.6 Hz, 1H), 1.77–1.68 (m, 5H), 1.45–1.34 (m, 5H), 1.06–0.98 (m, 2H); ^{13}C NMR (125 MHz, CD_3OD, referenced to CD_3OD): δ = 175.1, 175.0, 163.0, 162.7, 144.70, 144.66, 141.1, 141.04, 135.5, 134.86, 134.80, 129.87, 129.86, 129.00, 128.97, 128.92, 128.0, 127.9, 118.6, 95.0, 94.9, 61.3, 61.0, 50.7, 50.5, 49.6, 47.2, 47.1, 43.2, 43.1, 37.59, 37.56, 35.2, 33.5, 30.2, 26.9, 26.5, 23.1, 22.9; HRMS (ESI) calcd for $C_{29}H_{35}N_4O_2$ $[M+H]^+$: 471.2760. Found: 471.2760.

4.1.21. (*S*)-2-((((3*S*,4a*R*,8a*S*)-2-(4-Bromobenzoyl)decahydroisoquinolin-3-yl)methyl)amino)-3-(1*H*-imidazol-4-yl)propanal 23

A title compound **23** was synthesized from the de-tritylated product of **19** as above. Colorless oil; yield, 36%: $[\alpha]_D^{28}$ −1.1 (*c* 0.40, CH_3OH); ^1H NMR (400 MHz, CD_3OD, referenced to residual CH_3OH): δ = 8.72 (br s, 1H), 7.66–7.64 (m, 2H), 7.46 (br s, 1H), 7.41 (d, *J* = 8.4 Hz, 2H), 5.11–5.03 (m, 1H), 4.78 (dd, *J* = 11.0, 3.0 Hz, 1H), 3.85–3.75 (m, 2H), 3.47–3.39 (m, 2H), 3.26–3.24 (m, 1H), 2.96–2.84 (m, 1H), 1.78–1.54 (m, 5H), 1.43–1.22 (m, 5H), 1.07–0.93 (m, 2H); ^{13}C NMR (100 MHz, CD_3OD, referenced to CD_3OD): δ = δ = 174.1, 173.5, 163.1, 162.6, 135.7, 135.45, 135.39, 133.0, 130.39, 130.35, 130.2, 125.84, 125.78, 118.8, 118.7, 95.1, 94.9, 61.3, 61.0, 50.64, 50.58, 43.3, 43.2, 37.71, 37.70, 37.68, 35.25, 35.22, 33.7, 30.4, 30.3, 27.0, 26.6, 23.2, 23.0; HRMS (ESI) calcd for $C_{23}H_{30}BrN_4O_2$ $[M+H]^+$: 473.1552. Found: 473.1543.

4.1.22. (*S*)-2-[({(3*R*,4a*S*,8a*R*)-2-[(1,1′-Biphenyl)-4-carbonyl]decahydroisoquinolin-3-yl}methyl)amino]-3-(1*H*-imidazol-4-yl)propanal 24

A title compound **24** was synthesized from the de-tritylated product of **20** as above. Colorless oil; yield, 30%: $[\alpha]_D^{29}$ −2.3 (*c* 0.61, CH_3OH); ^1H NMR (500 MHz, CD_3OD, referenced to residual CH_3OH): δ = 8.76 (*s*, 1H), 7.74 (d, *J* = 6.4 Hz, 2H), 7.66 (d, *J* = 6.0 Hz, 2H), 7.56 (d, *J* = 6.4 Hz, 2H), 7.48–7.45 (m, 3.5H), 7.40–7.37 (m, 1.5H), 5.09 (br s, 1H), 3.86–3.75 (m, 2H), 3.64–3.59 (m, 1H), 3.55–3.48 (m, 1H), 3.35–3.32 (m, 1H), 3.28–3.26 (m, 1H), 2.93–2.91 (m, 1H), 1.79–1.66 (m, 5H), 1.47–1.28 (m. 5H), 1.07–0.97 (m, 2H); ^{13}C NMR (125 MHz, CD_3OD, referenced to CD_3OD): δ = 175.5, 175.4, 163.1, 162.8, 145.0, 141.2, 135.7, 135.6, 134.9, 130.1, 129.2, 129.1, 128.2, 128.1, 119.0, 95.4, 95.1, 62.1, 61.6, 50.8, 43.2, 43.1, 37.7, 35.5, 35.4, 33.8, 33.7, 30.4, 27.0, 26.6, 24.5, 24.1; LRMS (ESI) calcd for $C_{29}H_{35}N_4O_2$ $[M+H]^+$: 471.28. Found: 471.30.

4.1.23. (*S*)-2-({[(3*R*,4a*S*,8a*R*)-2-(4-Bromobenzoyl)decahydroisoquinolin-3-yl]methyl}amino)-3-(1*H*-imidazol-4-yl)propanal 25

A title compound **25** was synthesized from the de-tritylated product of **21** as above. Colorless oil; yield, 28%: $[\alpha]_D^{29}$ −7.8 (*c* 0.36, CH_3OH); ^1H NMR (500 MHz, CD_3OD, referenced to residual CH_3OH): δ = 8.72 (br s, 1H), 7.66–7.62 (m, 2H), 7.45 (s, 1H), 7.43–7.36 (m, 2H), 5.06 (m, 1H), 3.83–3.75 (m, 2H), 3.49–3.46 (m, 2H), 3.34–3.33 (m, 1H), 3.28–3.23 (m, 1H), 2.92–2.85 (m, 1H), 1.76–1.58 (m, 5H), 1.44–1.26 (m, 5H), 1.06–0.93 (m, 2H); ^{13}C NMR (125 MHz, CD_3OD, referenced to CD_3OD): δ = 174.43, 174.35, 163.1, 162.8, 135.7, 135.6, 135.3, 133.0, 130.3, 125.9, 118.8, 95.4, 95.1, 62.1, 61.6, 50.7, 43.1, 43.0, 37.7, 37.6, 35.4, 35.3, 33.7, 30.3, 27.0, 26.6, 24.6, 24.1; LRMS (ESI) calcd for $C_{23}H_{30}BrN_4O_2$ $[M+H]^+$: 473.16. Found: 473.25.

4.1.24. (1*S*,6*R*)-6-{2-[(4-Bromobenzyl)oxy]ethyl}cyclohex-3-enecarboxylic acid 31

To a solution of 1,3-butadiene (20 wt% solution in toluene, 108 mL, 255 mmol) was added (*E*)-ethyl 5-[(4-bromobenzyl)oxy]pent-2-enoate[32] (20.0 g, 63.9 mmol), and the mixture was heated at 225 °C for 60 h. After the reaction mixture was cooled to room temperature, water was added and the whole was extracted with AcOEt. The organic layer was washed with 1 M HCl and brine, dried over Na_2SO_4, filtered, and concentrated. The residue was purified by silica gel column chromatography (hexane/AcOEt = 35:1) to give an ethyl ester of **29**, (1*S*/*R*, 6*R*/*S*)-ethyl 6-{2-[(4-bromobenzyl)oxy]ethyl)}cyclohex-3-enecarboxylate, (11.7 g, 50%) as a yellow pale oil. ^1H NMR (400 MHz): δ = 7.47–7.45 (m, 2H), 7.22 (d, *J* = 8.4 Hz, 2H), 5.65 (m, 2H), 4.43 (dd, *J* = 18.8, 12.0 Hz, 2H) 4.14 (q, *J* = 7.2 Hz, 2H), 3.52–3.49 (m, 2H), 2.41–2.20 (m, 4H), 2.08–2.03 (m, 1H), 1.81–1.72 (m, 2H), 1.53–1.46 (m, 1H), 1.26 (t, *J* = 7.2 Hz, 3H); ^{13}C NMR (100 MHz): δ = 175.8, 137.5, 131.4, 129.2, 125.7, 124.8, 121.3, 72.1, 68.1, 60.3, 45.3, 33.7, 32.4, 29.9, 28.1, 14.3; HRMS (EI) Calcd for $C_{18}H_{23}BrO_3$ $[M]^+$: 366.0831. Found: 366.0826.

The above ester (31.8 g, 86.6 mmol) was dissolved in 2 M NaOH/THF (1:1, 100 mL). After being stirred for 15 h under reflux, the reaction mixture was cooled to room temperature. The mixture was acidified with 2 M HCl, and the whole was extracted with AcOEt. The organic layer was washed with brine, dried over Na_2SO_4, filtered, and concentrated. The residue purified by silica gel column chromatography (hexane/AcOEt = 3:1). The product was dissolved in AcOEt (300 mL) and then (*S*)-(−)-phenylethylamine (11 mL, 87 mmol) was added. After 12 h, the solid was collected by suction filtration. The free acid was liberated from the salt by treatment with 2 M HCl and extraction with AcOEt. The organic layer was washed with brine, dried over Na_2SO_4, filtered and concentrated. The residue was purified by silica gel column chromatography (hexane/AcOEt = 3:1) to give **31** [7.63 g, 26% (50% max.)] as a colorless oil. $[\alpha]_D^{28}$ +22 (*c* 0.78, $CHCl_3$); ^1H NMR (400 MHz): δ = 7.47–7.45 (m, 2H), 7.20 (d, *J* = 8.0 Hz, 2H), 5.69–5.66 (m, 2H), 4.44 (dd, *J* = 17.0, 12.2 Hz, 2H) 3.56–3.49 (m, 2H), 2.47–2.20 (m, 4H), 2.12–2.07 (m, 1H), 1.91–1.75 (m, 2H), 1.60–1.51 (m, 1H); ^{13}C NMR (100 MHz): δ = 181.1, 137.3, 131.5, 129.3, 125.7, 124.5, 121.4, 72.2, 68.0, 44.9, 33.6, 32.1, 29.5, 27.7; HRMS (EI) calcd for $C_{16}H_{19}BrO_3$ $[M]^+$: 338.0518. Found: 338.0520.

4.1.25. [(1*S*,6*R*)-6-{2-[(4-Bromobenzyl)oxy]ethyl}cyclohex-3-en-1-yl]methanol

To a solution of **31** (7.70 g, 22.7 mmol) in THF (80 mL), Et_3N (6.4 mL, 46 mmol) and IBCF (4.5 mL, 34 mmol) were added at −20 °C. After being stirred for 15 min at the same temperature, $NaBH_4$ (3.47 g, 91.2 mmol) and H_2O (10 drops from a pipette) was added. The mixture was warmed up to room temperature and then the reaction was quenched with saturated aqueous NH_4Cl. The whole was extracted with AcOEt and the organic layer was washed with brine and dried over Na_2SO_4, filtered, and concentrated. The residue was purified by silica gel column chromatography (hexane/AcOEt = 3:1) to give a title alcohol (5.68 g, 77%) as a colorless oil. $[\alpha]_D^{29}$ +22 (*c* 0.65, $CHCl_3$); ^1H NMR (400 MHz): δ = 7.48–7.46 (m, 2H), 7.20 (d, *J* = 8.4 Hz, 2H), 5.65–5.58 (m, 2H), 4.45 (s, 2H), 3.68 (dd, *J* = 10.8, 6.4 Hz, 1H), 3.62 (dd, *J* = 10.8, 5.2 Hz, 1H), 3.58–3.47 (m, 2H), 2.15–2.09 (m, 2H), 2.00–1.76 (m, 4H), 1.66–1.49 (m, 3H); ^{13}C NMR (100 MHz): δ = 137.4, 131.5, 129.3, 125.8, 125.5, 121.4, 72.3, 68.7, 65.0, 39.7, 32.9, 31.1, 29.5, 26.6; HRMS (EI) calcd for $C_{16}H_{21}BrO_2$ $[M]^+$: 324.0725. Found: 324.0732.

4.1.26. {[(1*S*,6*R*)-6-{2-[(4-Bromobenzyl)oxy]ethyl}cyclohex-3-en-1-yl]methoxy}(*tert*-butyl)diphenylsilane

TBDPS-Cl (5.0 mL, 19 mmol) was added to a solution of above alcohol (5.66 g, 17.4 mmol) and imidazole (1.43 g, 21.0 mmol) in CH_2Cl_2 (50 mL), and the mixture was stirred for 8 h at room temperature. The reaction was quenched with saturated aqueous NH_4Cl, and the whole was extracted with AcOEt. The organic layer was washed with brine, dried over Na_2SO_4, filtered, and concentrated. The residue was purified by silica gel column chromatography (hexane/AcOEt = 30:1) to give a di-protected alcohol compound (9.81 g, quant.) as a colorless oil. $[\alpha]_D^{28}$ +18.6 (*c* 1.74, $CHCl_3$); ^1H NMR (400 MHz): δ = 7.67–7.64 (m, 4H), 7.44–7.34 (m, 8H), 7.17 (d, *J* = 8.4 Hz, 2H), 5.63–5.54 (m, 2H), 4.41 (dd, *J* = 15.2, 12.0 Hz, 2H), 3.68 (dd, *J* = 9.8, 5.4 Hz, 1H), 3.62 (dd, *J* = 10.0, 6.8 Hz, 1H), 3.50–3.46 (m, 2H), 2.16–1.96 (m, 3H), 1.87–1.81 (m, 2H), 1.71–1.68 (m, 2H), 1.48–1.44 (m, 1H), 1.05 (s, 9H); ^{13}C NMR (100 MHz): δ = 137.7, 135.61, 135.60, 133.94, 133.92, 131.4, 129.5, 129.1, 127.6, 125.8, 125.3, 121.2, 72.1, 68.7, 65.9, 39.6, 32.9, 30.8, 29.0, 26.9, 26.7, 19.3; HRMS (FAB) Calcd. For $C_{32}H_{40}BrO_5$ $[M+H]^+$: 563.1981. Found: 563.1988.

4.1.27. 2-[(1*R*,2*S*)-2-{[(*tert*-Butyldiphenylsilyl)oxy]methyl}cyclohexyl]ethanol 32

To a solution of above di-protected alcohol (9.81 g, 17.4 mmol) in CH_3OH/EtOAc/saturated aqueous $NaHCO_3$ (5:5:1, 110 mL), Pd-C (3.8 g) was added, and the mixture was stirred under a hydrogen gas atmosphere at room temperature for 6 h. The mixture was filtered through Celite and a silica gel layer, and the filtrate was dried over Na_2SO_4, filtered, and concentrated. The residue was purified by silica gel column chromatography (hexane/AcOEt = 6:1) to give **32** (6.90 g, quant.) as a colorless oil. $[\alpha]_D^{28}$ +12 (*c* 0.65, $CHCl_3$); ^1H NMR (400 MHz): δ = 7.68–7.65 (m, 4H), 7.43–7.36 (m, 6H), 3.67–3.56 (m, 4H), 1.78–1.70 (m, 5H), 1.37–1.21 (m, 6H), 1.06 (s, 9H), 1.02–0.96 (m, 1H); ^{13}C NMR (100 MHz): δ = 135.69, 135.66, 133.91, 133.89, 129.55, 129.54, 127.60, 127.57, 66.5, 61.0, 44.5,

36.5, 35.5, 31.9, 30.0, 26.9, 26.1, 26.0, 19.3; HRMS (FAB) calcd for $C_{25}H_{37}O_2Si$ $[M+H]^+$: 397.2563. Found: 397.2558.

4.1.28. (*S*)-2-[({(3*S*,4a*R*,8a*S*)-2-[(1,1′-Biphenyl)-4-carbonyl]decahydroisoquinolin-3-yl}methyl)amino]-3-(1*H*-imidazol-4-yl)propanal 40

Title compound was prepared from **32** according to the same procedure[33] employed for the synthesis of **22** starting from enantiomer mixture **7**. Colorless solid; yield, 30%: $[\alpha]_D^{28}$ −4.3 (*c* 0.83, CH_3OH); ^1H NMR (500 MHz, CD_3OD, referenced to residual CH_3OH): δ = 8.81 (br s, 1H), 7.75–7.73 (m, 2H), 7.67–7.65 (m, 2H), 7.58 (d, *J* = 8.0 Hz, 2H), 7.51 (br s, 1H), 7.48–7.45 (m, 2.5H), 7.40–7.36 (m, 1.5H), 5.14–5.13 (m, 1H), 4.81 (dd, *J* = 9.8, 2.6 Hz, 1H), 3.89–3.80 (m, 2H), 3.63–3.59 (m, 1H), 3.44–3.39 (m, 1H), 3.34 (s, 1H), 2.97 (t, *J* =12.6 Hz, 1H), 1.82–1.62 (m, 5H), 1.45–1.28 (m, 5H), 1.09–0.89 (m, 2H); ^{13}C NMR (125 MHz, CD_3OD, referenced to CD_3OD): δ = 175.24, 175.17, 163.2, 162.8, 144.9, 144.8, 141.21, 141.20, 135.6, 135.03, 134.97, 130.1, 129.22, 129.18, 129.12, 128.2, 128.1, 118.98, 118.95, 95.0, 94.9, 61.3, 60.9, 50.73, 50.69, 49.8, 47.1, 47.0, 43.33, 43.30, 37.8, 37.7, 35.4, 33.7, 30.4, 27.0, 26.6, 23.1, 22.9; HRMS (ESI) Calcd. For $C_{29}H_{35}N_4O_2$ $[M+H]^+$: 471.2760. Found: 471.2765.

Compounds **41**, **44**, and **45–49** listed in Table 1 were similarly prepared as above.

4.1.29. Compound 41

4.1.29.1. (S)-2-({[(3S,4aR,8aS)-2-(4-Bromobenzoyl)decahydroisoquinolin-3-yl]methyl}amino)-3-(1H-imidazol-4-yl)-propanal 41. Colorless solid; yield, 23%: $[\alpha]_D^{28}$ −0.64 (*c* 0.88, CH_3OH); ^1H NMR (400 MHz, CD_3OD, referenced to residual CH_3OH): δ = 8.80 (br s, 1H), 7.65–7.63 (m, 2H), 7.49 (br s, 1H), 7.42 (d, *J* = 8.4 Hz, 2H), 5.11 (m, 1H), 4.81–4.78 (m, 1H), 3.86–3.78 (m, 2H), 3.47–3.34 (m, 2H), 2.97–2.91 (m, 1H), 1.75–1.60 (m, 5H), 1.42–1.24 (m, 5H), 1.04–0.96 (m, 2H); ^{13}C NMR (100 MHz, CD_3OD, referenced to CD_3OD): δ = 174.2, 174.1, 163.2, 162.8, 135.6, 135.44, 135.39, 132.9, 130.40, 130.36, 130.0, 125.8, 125.7, 119.0, 118.9, 95.0, 94.9, 61.2, 60.8, 50.64, 50.60, 43.24, 43.22, 37.69, 37.66, 35.25, 35.23, 33.7, 30.4, 30.3, 27.0, 26.6, 23.1, 22.9; HRMS (ESI) calcd for $C_{23}H_{30}BrN_4O_2$ $[M+H]^+$: 473.1552. Found: 473.1546.

4.1.30. Compound 44

Colorless solid; yield, 31%: $[\alpha]_D^{28}$ −1.62 (*c* 1.23, CH_3OH); ^1H NMR (500 MHz, CD_3OD, referenced to residual CH_3OH): δ = 8.75 (s, 1H), 7.75 (d, *J* = 8.0 Hz, 2H), 7.66 (d, *J* = 7.2 Hz, 2H), 7.57 (d, *J* = 8.0 Hz, 2H), 7.47–7.45 (m, 3.5H), 7.40–7.37 (m, 1.5H), 5.09 (br s, 1H), 3.80 (m, 2H), 3.66–3.63 (m, 1H), 3.51 (m, 1H), 3.26 (m, 1H), 2.93–2.91 (m, 1H), 1.77–1.68 (m, 5H), 1.45–1.35 (m, 5H), 1.07–0.97 (m, 2H); ^{13}C NMR (125 MHz, CD_3OD, referenced to CD_3OD): δ = 175.5, 175.4, 163.2, 162.8, 144.9, 141.2, 135.6, 135.5, 134.9, 130.1, 129.2, 129.1, 128.2, 128.1, 119.1, 95.2, 95.0, 62.0, 61.4, 50.8, 43.12, 43.10, 37.73, 37.69, 35.5, 35.4, 33.7, 30.4, 27.0, 26.6, 24.4, 23.9; HRMS (ESI) calcd for $C_{29}H_{35}N_4O_2$ $[M+H]^+$: 471.2760. Found: 471.2756.

4.1.31. Compound 45

Colorless solid; yield, 30%: $[\alpha]_D^{28}$ −6.1 (*c* 1.0, CH_3OH); ^1H NMR (500 MHz, CD_3OD, referenced to residual CH_3OH): δ = 8.82 (br s, 1H), 7.65 (d, *J* = 8.4 Hz, 2H), 7.49 (s, 1H), 7.40 (d, *J* = 8.0 Hz, 2H), 5.06 (m, 1H), 4.83 (m, 1H), 3.86–3.76 (m, 2H), 3.51–3.43 (m, 2H), 3.27–3.25 (m, 1H), 2.93–2.90 (m, 1H), 1.76–1.66 (m, 5H), 1.44–1.30 (m, 5H), 1.04–0.96 (m, 2H); ^{13}C NMR (125 MHz, CD_3OD, referenced to CD_3OD): δ = 174.43, 174.35, 163.1, 162.8, 135.6, 135.5, 135.3, 133.0, 130.4, 125.9, 119.1, 95.3, 95.0, 61.8, 61.3, 50.7, 43.03, 43.01, 37.7, 37.6, 35.4, 35.3, 33.7, 30.33, 30.31, 27.0, 26.6, 24.4, 23.9; HRMS (ESI) calcd for $C_{23}H_{30}BrN_4O_2$ $[M+H]^+$: 473.1552. Found: 4731537. Found: 4731537.

4.1.32. Compound 46

Colorless solid; yield, 31%: $[\alpha]_D^{29}$ −6.7 (*c* 0.10, CH_3OH); ^1H NMR (400 MHz, CD_3OD, referenced to residual CH_3OH): δ = 8.66 (br s, 1H), 7.79–7.73 (m, 2H), 7.64–7.55 (m, 4H), 7.48–7.45 (m, 3.5H), 7.41–7.37 (m, 1.5H), 5.19–5.18 (m, 1H), 4.77 (dd, *J* = 12.6, 3.2 Hz, 1H), 3.87–3.79 (m, 2H), 3.63–3.58 (m, 1H), 3.46–3.40 (m, 1H), 3.36–3.34 (m, 0.5H), 3.25–3.23 (m, 1.5H), 2.99–2.93 (m, 1H), 1.79–1.62 (m, 5H), 1.42–1.21 (m, 5H), 1.10–0.89 (m, 2H); LRMS (ESI) calcd for $C_{29}H_{35}N_4O_2$ $[M+H]^+$: 471.28. Found: 471.35.

4.1.33. Compound 47

Colorless solid; yield, 27% (obtained as the mixture of a diastereomer derived from Pd-mediated cyclization): ^1H NMR (400 MHz, CD_3OD, referenced to residual CH_3OH): δ = 8.84 (br s, 1H), 7.59–7.38 (m, 11H), 5.03 (m, 1H), 4.81 (dd, *J* = 10.0, 2.8 Hz, 1H), 3.90 (m, 1H), 3.69–3.59 (m, 1H), 3.41 (m, 1H), 3.34 (s, 1H), 3.27–3.24 (m, 1H), 2.93–2.87 (m, 1H), 2.61–2.54 (m, 1H), 1.59–1.56 (m, 2H), 1.50 (d, *J* = 10.4 Hz, 1H), 1.42 (d, *J* = 12.4 Hz, 1H), 1.18–1.08 (m, 2H), 0.99–0.88 (m, 3H), 0.70–0.61 (m, 1H), 0.46–0.44 (m, 1H); LRMS (ESI) calcd for $C_{29}H_{35}N_4O_2$ $[M+H]^+$: 471.28. Found: 471.35.

4.1.34. Compound 48

Colorless solid; yield, 25%: $[\alpha]_D^{29}$ −3.7 (*c* 0.15, CH_3OH); ^1H NMR (400 MHz, CD_3OD, referenced to residual CH_3OH): δ = 8.61 (br s, 1H), 7.52–7.46 (m, 6H), 7.45–7.41 (m, 1H), 5.13–5.11 (m, 2H), 4.77 (dd, *J* = 11.8, 3.4 Hz, 1H), 3.79–3.66 (m, 1H), 3.56–3.50 (m, 1H), 3.42–3.32 (m, 1H), 3.26–3.20 (m, 1H), 2.97–2.91 (m, 1H), 1.78–1.59 (m, 5H), 1.40–1.20 (m, 5H), 1.08–0.86 (m, 2H); LRMS (ESI) calcd for $C_{23}H_{31}N_4O_2$ $[M+H]^+$: 395.24. Found: 395.30.

4.1.35. Compound 49

Colorless solid; yield, 18%: $[\alpha]_D^{29}$ −3.6 (*c* 0.18, CH_3OH); ^1H NMR (400 MHz, CD_3OD, referenced to residual CH_3OH): δ = 8.68 (br s, 1H), 7.56–7.53 (m, 2H), 7.44 (br s, 1H), 7.25–7.19 (m, 3H), 5.10 (m, 1H), 4.77 (dd, *J* = 11.4, 3.2 Hz, 1H), 3.84–3.75 (m, 2H), 3.52–3.47 (m, 1H), 3.40–3.34 (m, 1H), 3.25–3.23 (m, 1H), 2.96–2.89 (m, 1H), 1.78–1.65 (m, 5H), 1.43–1.23 (m, 5H), 1.05–0.93 (m, 2H); LRMS (ESI) calcd for $C_{23}H_{30}FN_4O_2$ $[M+H]^+$: 413.24. Found: 413.35.

4.2. Estimation of IC_{50} values

Peptide substrate [H-Thr-Ser-Ala-Val-Leu-Gln-Ser-Gly-Phe-Arg-Lys-NH_2][28] (111 μM) in a reaction solution (25 μL of 20 mM Tris–HCl buffer pH 7.5 containing 7 mM DTT) was incubated with the R188I SARS $3CL^{pro}$[28] (56 nM) at 37 °C for 60 min in the presence of various inhibitor concentrations at 37 °C for 60 min. The cleavage reaction was monitored by analytical HPLC [Cosmosil 5C18 column (4.6 × 150 mm), a linear gradient of CH_3CN (10–20%) in an aq0.1% TFA over 30 min], and the cleavage rates were calculated from the reduction in the substrate peak area. Each IC_{50} value was obtained from the sigmoidal dose-response curve (see Fig. S1 for a typical sigmoidal curve). Each experiment was repeated 3 times and the results were averaged.

4.3. X-ray crystallography

The purified SARS $3CL^{pro}$ in 20 mM Bis–Tris pH 5.5, 10 mM NaCl, and 1 mM DTT was concentrated to 8 mg/mL.[13] Crystals of SARS $3CL^{pro}$ were grown at 4 °C using a sitting-drop vapor diffusion method by mixing it with an equal volume of reservoir solution containing 100 mM MES pH 6.2, 5–10% PEG20000, and 5 mM DTT. Cubic-shaped crystals with dimensions of 0.3 mm × 0.3 mm × 0.3 mm grew within 3 days. The crystals were then soaked for 24 h with reservoir-based solution of 100 mM MES

pH 6.2, 5–8% PEG20000, and 5 mM DTT containing 3 mM of **40** or **44**. Crystals were then transferred into a cryobuffer of 100 mM MES pH 6.2, 10% PEG20000, 5 mM DTT, 15% ethylene glycol containing 3 mM of **40** or **44**, and flash-frozen in a nitrogen stream at 100 K. X-ray diffraction data of SARS $3CL^{pro}$ in complexes with inhibitor **40** or **44** were collected at the SPring-8, beamline BL44XU with a Rayonix MX300HE CCD detector at a wavelength of 0.900 Å.

Crystals of SARS $3CL^{pro}$ in a complex with **41** were obtained by co-crystallization using sitting-drop vapor diffusion at 4 °C and mixing an equal volume of protein-inhibitor complex (final inhibitor concentration of 3 mM) and a reservoir solution containing 100 mM MES pH 6.0, 5–6% PEG20000, and 5 mM DTT. Cubic-shaped crystals with dimensions of 0.2 mm × 0.2 mm × 0.2 mm were obtained within 3 days. Crystals were transferred into cryobuffer with 100 mM MES pH 6.0, 6% PEG20000, 5 mM DTT, 15% ethylene glycol, and 3 mM of **41** and then flash-frozen in a nitrogen stream at 100 K. X-ray diffraction data were collected on a Rigaku RAXIS VII imaging-plate detector at a wavelength of 1.5418 Å equipped with an in-house rotating anode FR-E/Super Bright X-ray generator and Confocal VariMax (VariMax HF) optics system.

The structures of SARS $3CL^{pro}$ in a complex with inhibitors were determined by molecular replacement using the Molrep[34] program with a R188I SARS $3CL^{pro}$ structure (PDB code 3AW1[13]) as the search model. Rigid body refinement and subsequent restrained refinement protocols were performed with the program Refmac 5[35] of the CCP package.[36] The Coot program[37] was used for manual model rebuilding. Water molecules were added using Coot only after the refinement of protein structures had converged. Ligands generated on JLigand[38] software were directly built into the corresponding difference in electron density, and the model was then subjected to an additional round of refinement. The figures for structural representation were generated on Pymol[39] or chimera[40] software.

5. PDB ID codes

4TWY, 4TWW, and 4WY3.

Acknowledgments

This work was supported, in part, by a Grant-in-aid for Scientific Research 25460160 to K.A. from the Japan Society for the Promotion of Science and by a grant for Adaptable and Seamless Technology Transfer Program through Target-driven R&D AS251Z01976Q to Y.H. from Japan Science and Technology Agency. We thank Shimadzu Co. for the measurements of mass spectra using LCMS-IT-TOF MS.

Supplementary data

Supplementary data (the HPLC data for the evaluation of purities using a reversed-phase or chiral column, typical sigmoidal curves used to obtain IC_{50} values, and NMR data of synthesized compounds) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2014.12.028.

References and notes

1. Lee, N.; Hui, D.; Wu, A.; Chan, P.; Cameron, P.; Joynt, F. M.; Ahuja, A.; Yung, M. Y.; Leung, C. B.; To, K. F.; Leu, M. D.; Szeto, C. C.; Chung, S.; Sung, J. J. Y. *N. Engl. J. Med.* **1986**, *2003*, 348.
2. Drosten, C.; Günther, S.; Preiser, W.; Ven der Werf, S.; Brodt, H. R.; Becker, S.; Rabenau, H.; Panning, M.; Kolensnikova, L.; Fouchier, R. A. M.; Berger, A.; Burguière, A. M.; Cinatl, J.; Eickmann, M.; Escriou, N.; Grywna, K.; Kramme, S.; Manuguerra, J.; Müller, S.; Rickerts, V.; Stürmer, M.; Vieth, S.; Klenk, H. D.; Osterhaus, A. D. M. E.; Schmitz, H.; Doerr, H. W. *N. Engl. J. Med.* **1967**, *2003*, 348.
3. Ksiazek, T. G.; Erdman, D.; Goldsmith, C. S.; Zaki, S. R.; Peret, T.; Emery, S.; Tong, S.; Urbani, C.; Comer, J. A.; Lim, W.; Rollin, P. E.; Dowell, S. F.; Ling, A. E.; Humphrey, C. D.; Shieh, W. J.; Guarner, J.; Paddock, C. D.; Rota, P.; Fields, B.; DeRisi, J.; Yang, J. Y.; Cox, N.; Hughes, J. M.; LeDuc, J. W.; Bellini, W. J.; Anderson, L. J. *N. Engl. J. Med.* **1953**, *2003*, 348.
4. Li, W.; Shi, Z.; Yu, M.; Ren, W.; Smith, C.; Epstein, J. H.; Wang, H.; Crameri, G.; Hu, Z.; Zhang, J.; McEachern, J.; Field, H.; Daszak, P.; Eaton, B. T.; Zhang, S.; Wang, L. F. *Science* **2005**, *310*, 676.
5. Lau, S. K. P.; Woo, P. C. Y.; Li, K. S. M.; Huang, Y.; Tsoi, H. W.; Wong, B. H. L.; Wong, S. S. Y.; Leung, S. Y.; Chan, K. H.; Yuen, K. Y. *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102*, 14040.
6. Ren, Z.; Yan, L.; Zhang, N.; Guo, Y.; Yang, C.; Lou, Z.; Rao, Z. *Protein Cell* **2013**, *4*, 248.
7. Kilianski, A.; Mielech, A. M.; Deng, X.; Baker, S. *J. Virol.* **2013**, *87*, 11955.
8. Rota, P. A.; Oberste, M. S.; Monroe, S. S.; Nix, W. A.; Campagnoli, R.; Icenogle, J. P.; Penaranda, S.; Bankamp, B.; Maher, K.; Chem, M. H.; Tong, W.; Tamin, A.; Lowe, L.; Frace, M.; DeRisi, J. L.; Chen, Q.; Wang, D.; Erdman, D. D.; Peret, T. C.; Burns, C.; Ksiazek, T. G.; Rollin, P. E.; Sanchez, A.; Liffick, S.; Holloway, B.; Limor, J.; McCaustland, K.; Olsen-Rasmussen, M.; Fouchier, R.; Gunther, S.; Osterhaus, A. D.; Drosten, C.; Pallansch, M. A.; Anderson, L. J.; Bellini, W. J. *Science* **2003**, *300*, 1394.
9. Marra, M. A.; Jones, S. J.; Astell, C. R.; Holt, R. A.; Brooks-Wilson, A.; Butterfield, Y. S.; Khattra, J.; Asano, J. K.; Barber, S. A.; Chan, S. Y.; Cloutier, A.; Coughlin, S. M.; Freeman, D.; Girn, N.; Griffith, O. L.; Leach, S. R.; Mayo, M.; McDonald, H.; Montgomery, S. B.; Pandoh, P. K.; Petrescu, A. S.; Robertson, A. G.; Schein, J. E.; Siddiqui, A.; Smailus, D. E.; Stott, J. M.; Yang, G. S.; Plummer, F.; Andonov, A.; Artsob, H.; Bastien, N.; Bernard, K.; Booth, T. F.; Bowness, D.; Czub, M.; Drebot, M.; Fernando, L.; Flick, R.; Garbutt, M.; Gray, M.; Grolla, A.; Jones, S.; Feldmann, H.; Meyers, A.; Kabani, A.; Li, Y.; Normand, S.; Stroher, U.; Tipples, G. A.; Tyler, S.; Vogrig, R.; Ward, D.; Watson, B.; Brunham, R. C.; Krajden, M.; Petric, M.; Skowronski, D. M.; Upton, C.; Roper, R. L. *Science* **2003**, *300*, 1399.
10. Thiel, V.; Ivanov, K. A.; Putics, Á.; Hertzig, T.; Schelle, B.; Bayer, S.; Weißbrich, B.; Snijder, E. J.; Rabenau, H.; Doerr, H. W.; Gorbalenya, A. E.; Ziebuhr, J. *J. Gen. Viol.* **2003**, *84*, 2305.
11. Anand, K.; Ziebuhr, J.; Wadhwani, P.; Mesters, J. R.; Hilgenfeld, R. *Science* **2003**, *300*, 1763.
12. Fan, K.; Wei, P.; Feng, Q.; Chen, S.; Huang, C.; Ma, L.; Lai, B.; Pei, J.; Liu, Y.; Chen, J.; Lai, L. *J. Biol. Chem.* **2004**, *279*, 1637.
13. Akaji, K.; Konno, H.; Mitsui, H.; Teruya, K.; Shimamoto, Y.; Hattori, Y.; Ozaki, T.; Kusunoki, M.; Sanjoh, A. *J. Med. Chem.* **2011**, *54*, 7962. and references cited therein.
14. Thanigaimalai, P.; Konno, S.; Yamamoto, T.; Koiwai, Y.; Taguchi, A.; Takayama, K.; Yakushiji, F.; Akaji, K.; Kiso, Y.; Kawasaki, Y.; Chen, S.-E.; Naser-Tavakolian, A.; Schön, A.; Freire, E.; Hayashi, Y. *Eur. J. Med. Chem.* **2013**, *65*, 436.
15. Chuck, C.-P.; Chen, C.; Ke, Z.; Wan, D. C.-C.; Chow, H.-F.; Wong, K.-B. *Eur. J. Med. Chem.* **2013**, *59*, 1.
16. Regnier, T.; Sarma, D.; Hidaka, K.; Bacha, U.; Freire, E.; Hayashi, Y.; Kiso, Y. *Bioorg. Med. Chem. Lett.* **2009**, *19*, 2722.
17. Ghosh, A. K.; Xi, K.; Grum-Tokars, V.; Xu, X.; Ratia, K.; Fu, W.; Houser, K. V.; Baker, S. C.; Johnson, M. E.; Mesecar, A. D. *Bioorg. Med. Chem. Lett.* **2007**, *17*, 5876.
18. Park, J.-Y.; Kim, J. H.; Kwon, J. M.; Kwon, H.-J.; Jeong, H. J.; Kim, Y. M.; Kim, D.; Lee, W. S.; Ryu, Y. B. *Bioorg. Med. Chem.* **2013**, *21*, 3730.
19. Ryu, Y. B.; Park, S.-J.; Kim, Y. M.; Lee, J.-Y.; Seo, W. D.; Chang, J. S.; Park, K. H.; Rho, M.-C.; Lee, W. S. *Bioorg. Med. Chem. Lett.* **1873**, *2010*, 20.
20. Wen, C.-C.; Kuo, Y.-H.; Jan, J.-T.; Liang, P.-H.; Wang, S.-Y.; Liu, H.-G.; Lee, C.-K.; Chang, S.-T.; Kuo, C.-J.; Lee, S.-S.; Hou, C.-C.; Hsiao, P.-W.; Chien, S.-C.; Shyur, L.-F.; Yang, N.-S. *J. Med. Chem.* **2007**, *50*, 4087.
21. Ikejiri, M.; Saijo, M.; Morikawa, S.; Fukushi, S.; Mizutani, T.; Kurane, I.; Maruyama, T. *Bioorg. Med. Chem. Lett.* **2007**, *17*, 2470.
22. Cho, J. H.; Bernard, D. L.; Sidwell, R. W.; Kern, E. R.; Chu, C. K. *J. Med. Chem.* **2006**, *49*, 1140.
23. Biot, C.; Daher, W.; Chavain, N.; Fandeur, T.; Khalife, J.; Dive, D.; Clercq, E. D. *J. Med. Chem.* **2006**, *49*, 2845.
24. Liu, W.; Zhu, H.-M.; Niu, G.-J.; Shi, E.-Z.; Chen, J.; Sun, B.; Chen, W.-Q.; Zhou, H.-G.; Yang, C. *Bioorg. Med. Chem.* **2014**, *22*, 292.
25. Lee, H.; Mittal, A.; Patel, K.; Gatuz, J. L.; Truong, L.; Torres, J.; Mulhearn, D. C.; Johnson, M. E. *Bioorg. Med. Chem.* **2014**, *22*, 167.
26. Jacobs, J.; Grum-Tokars, V.; Zhou, Y.; Turlington, M.; Saldanha, S. A.; Chase, P.; Eggler, A.; Dawson, E. S.; Baez-Santos, Y. M.; Tomar, S.; Mielech, A. M.; Baker, S. C.; Lindsley, C. W.; Hodder, P.; Mesecar, A.; Stauffer, S. R. *J. Med. Chem.* **2013**, *56*, 534.
27. Ramajayam, R.; Tan, K.-P.; Liu, H.-G.; Liang, P.-H. *Bioorg. Med. Chem.* **2010**, *18*, 7849.
28. Akaji, K.; Konno, H.; Onozuka, M.; Makino, A.; Saito, H.; Nosaka, K. *Bioorg. Med. Chem.* **2008**, *16*, 9400.
29. Makabe, H.; Looi, K. K.; Hirota, M. *Org. Lett.* **2003**, *5*, 27.
30. Azzena, F.; Calvani, F.; Crotti, P.; Gardelli, C.; Macchia, F.; Pineschi, M. *Tetrahedron* **1995**, *51*, 10601.
31. Crane, S. N.; Black, W. C.; Palmer, J. T.; Davis, D. E.; Setti, E.; Robichaud, J.; Paquet, J.; Oballa, R. M.; Bayly, C. I.; McKay, D. J.; Somoza, J. R.; Chauret, N.; Seto, C.; Scheigetz, J.; Wesolowski, G.; Massé, F.; Desmarais, S.; Ouellet, M. *J. Med. Chem.* **2006**, *49*, 1066.
32. Synthesis of (*E*)-ethyl 5-[(4-bromobenzyl)oxy]pent-2-enoate is included in Supplementary data.
33. Procedures and data for the syntheses of **40** from **32** as well as the related compounds **41**, **44**, and **45**–**49** are included in Supplementary data.
34. Vagin, A.; Teplyakov, A. *Acta Crystallogr. D Biol. Crystallogr.* **2010**, *66*, 22.

35. Murshudov, G. N.; Vagin, A. A.; Dodson, E. J. *Acta Crystallogr. D Biol. Crystallogr.* **1997**, *53*, 240.
36. Winn, M. D.; Ballard, C. C.; Cowtan, K. D.; Dodson, E. J.; Emsley, P.; Evans, P. R.; Keegan, R. M.; Krissinel, E. B.; Leslie, A. G. W.; McCoy, A.; McNicholas, S. J.; Murshudov, G. N.; Pannu, N. S.; Potterton, E. A.; Powell, H. R.; Read, R. J.; Vagin, A.; Wilson, K. S. *Acta Crystallogr. D Biol. Crystallogr.* **2011**, *67*, 235.
37. Emsley, P.; Cowtan, K. *Acta Crystallogr. D Biol. Crystallogr.* **2004**, *60*, 2126.
38. Lebedev, A. A.; Young, P.; Isupov, M. N.; Moroz, O. V.; Vagin, A. A.; Murshudov, G. N. *Acta Crystallogr. D Biol. Crystallogr.* **2012**, *68*, 431.
39. The PyMOL Molecular Graphics System, Version 1.5.0.4 Schrödinger LLC.
40. Pettersen, E. F.; Goddard, T. D.; Huang, C. C.; Couch, G. S.; Greenblatt, D. M.; Meng, E. C.; Ferrin, T. E. *J. Comput. Chem.* **2004**, *25*, 1605.

蛋白質科学会アーカイブ, **7**, e074 (2014)

マウス神経芽細胞腫細胞由来の Proteinase K 耐性プリオン蛋白質 (PrPres)の検出

東北大学大学院・医学系研究科・神経化学分野

倉橋　洋史、逆瀬川　裕二、堂浦　克美

Detection of Proteinase K-resistant prion protein (PrPres) in mouse neuroblastoma cells

Department of Neurochemistry, Tohoku University Graduate School of Medicine
Hiroshi Kurahashi, Yuji Sakasegawa, Katsumi Doh-ura

（投稿日 2014/2/4、再投稿日 2014/4/4、受理日 2014/4/11）

キーワード：prion、PrPres、Proteinase K、neuroblastoma cell

概要

狂牛病の原因として知られているプリオンは、核酸を必要としない特異な感染体である。プリオンの感染性はプリオン蛋白質の立体構造が変化（異常化）することが原因と考えられている。正常型のプリオン蛋白質も異常型のプリオン蛋白質も一次構造が同じためにSDS-PAGE とウエスタンブロット法を用いた通常の蛋白質の検出では、両者を区別することはできない。そこで、異常型プリオン蛋白質のみを検出するために、異常型プリオン蛋白質が Proteinase K の分解に対して部分的に抵抗性を持つことと、その抵抗性蛋白質が易沈殿性であることを利用する。本稿では、プリオンが持続的に感染している神経芽細胞腫細胞由来の Proteinase K 耐性プリオン蛋白質(PrPres)を検出する一連の手法を紹介する。この手法では培養細胞から蛋白質の抽出後、1 日で PrPres を検出することができる。

イントロダクション

プリオンは牛海綿状脳症（Bovine Spongiform Encephalopathy, BSE）に代表されるプリオン病、伝達性（伝染性）海綿状脳症（Transmissible Spongiform Encephalopathy, TSE）を引き起こす(1)。プリオン病はウシの他、ヒト、ヒツジ、マウス、ハムスター、ネコ、シカ、ミンクなどの哺乳類で発症することが知られており、致死性の脳神経疾患である。(1)。代表的なヒトプリオン病のクロイツフェルト・ヤコブ病(Creutzfeldt-Jakob disease: CJD)には３つの病因があり、原因不明の孤発性 CJD、プリオン蛋白質をコードする遺伝子に変異があることで発症しやすくなる家族性 CJD、プリオンの感染により発症する獲得性 CJD に分類される(1)。動物種や病因のタイプに限らず、いずれのプリオン病も原因はプリオン蛋白質のコンフォメーションの変化によるものと考えられている。プリオン蛋白質は神経細胞で常に産生されているが、正常型ではプリオン病を引き起こさず、異常型になることでプリオン病を引き起こす。一次構造が同じプリオン蛋白質の正常型と異常型を見分ける方法として、Proteinase K の分解に対する抵抗性の違いを利用する方法がある(1)。異常型プリオン蛋白質（つまり、プリオン）は Proteinase K に対して部分

的に抵抗性を持つ。そして脳組織から Proteinase K 耐性の PrP を検出することが、プリオン病の診断基準の１つになっている。一方、マウス神経芽細胞腫 Neuro2A (N2a)細胞にプリオンを感染させ、細胞を継代しても持続的にプリオンを維持しているプリオン持続感染細胞が細胞研究で広く使われている(2,3)。例えば、プリオンに対する薬剤のスクリーニングや、プリオン増幅に関与する内在性因子の解析で利用されている(4-8)。動物実験を行うよりも安価で短時間で解析できる点、そして動物愛護の観点において、使用する実験動物数の数を減らせる点で、プリオン持続感染細胞を使用するメリットは大きい。本稿では、このプリオン持続感染細胞の PrPres を検出する一連の手法を紹介する(7,8)。

用語の説明

プリオンがつく単語には「プリオン」、「プリオン蛋白質」、「正常型プリオン蛋白質」、「異常型プリオン蛋白質」、「Proteinase K 耐性プリオン蛋白質」と様々あり、混乱を生じやすいので、この欄で紹介する。「プリオン蛋白質」は遺伝子 *PRNP* がコードする蛋白質の名前であり、一般的に PrP と表記される。プリオン蛋白質は、特に脳で強く発現しているが様々な組織の細胞でも発現している。名前にプリオンがつくために誤解されやすいのだが、「プリオン蛋白質」自身は、病原性や感染性を意味するものではない。病原性や感染性をもつ意味で使用される「プリオン」はプリオン蛋白質の立体構造が異常型になって、正常型のプリオン蛋白質を異常型に変換する能力を持つ。ヒツジのプリオン病名、スクレイピー(scrapie) から、動物種に関係なく、プリオンは PrPsc と表記され、正常型プリオン蛋白質は PrPc (cellular prion protein)と表記される。一方、プリオン持続感染細胞の異常型プリオン蛋白質は Proteinase K に対して抵抗性を持つことから PrPres (protease-resistant prion protein)と表記される。PrPsc も PrPres も「異常型プリオン蛋白質」ではあるが、PrPsc は感染性を持つ意味で使われ、PrPres は Proteinase K 耐性を持つ意味で使われており、PrPres は必ずしも感染性を意味するわけではない。Proteinase K に感受性がありながら感染性を示すプリオンが存在するという報告や、in vitro で増幅した異常型蛋白質は Proteinase K 耐性だが、必ずしも感染性を持たないという報告がある(9,10)。

実験の原理

プリオン持続感染細胞の細胞内にある PrP は全てが PrPres ということではなく、PrPc も含まれる。この両者は一次構造が同じために、SDS-PAGE で区別することはできない。そこで、PrPres のみを検出するためには、SDS-PAGE を行う前に全 PrP に含まれる PrPc を取り除かなければならない。そのために、本実験では検体の Proteinase K (PK) 処理と遠心分離を行う。PrPres は PK 処理によって N 末端が切断されるが、おおよそ 90 アミノ酸残基以降の C 末端は部分的に PK 抵抗性を持つために、N 末端を欠いた PrP が残る。この N 末端欠損型 PrP は特定の界面活性剤存在中の試験管内で（アミロイドを形成しているかは不明であるが、）容易に沈殿する性質を持つようになる。従って、超遠心機を用いなくても N 末端欠損型 PrP は遠心分離で沈殿し、(PK 処理は PrPc が完全に消化される条件ではあるが、)PrPc が PK 処理で完全に切断しきれなかったとしても PrPc は上清画分に分離されて取り除かれる。そして、沈殿画分を SDS-PAGE とウエスタンブロットを行うことで、PrPres を検出することができる。以上のことから、本実験の PK 処理はアミロイドにみられる PK 耐性の蛋白質を検出するという単純なものではない。本実験の PK

処理は PrPc を分解させるだけではなく、PrPres の沈殿性を高めるための処理でもあるという点で、他のアミロイド蛋白質の検出とは異なる。

装置・器具・試薬
CO_2インキュベーター
安全キャビネット
アスピレーター
細胞培養用(6 well) plate
冷却遠心機（最大遠心力 20,000 x g）
SDS-PAGE を行うにあたり必要な機器（電気泳動槽、泳動プレート、パワーサプライ）
セミドライトランスファー装置
イメージング装置、あるいは X 線フィルムと現像機
PVDF 膜
3MM 紙
セーフロックチューブ
PBS(-)
細胞溶解液(PBS(-), 0.5% NP40, 0.5% デオキシコール酸ナトリウム)
1 mg/mL Proteinase K
0.1 M フッ化フェニルメチルスルホニル(PMSF)
SDS-PAGE 用サンプルバッファー（25 mM Tris-HCl pH6.8, 1% SDS, 0.05% ブロモフェノールブルー, 4% グリセロール, 140 mM 2-メルカプトエタノール）
SDS-PAGE 用泳動バッファー（25 mM Tris, 192 mM glycine, 0.1% SDS）
トランスファーバッファー（陽極液 1 300 mM Tris, 20% メタノール; 陽極液 2 25 mM Tris, 20% メタノール; 陰極液 25 mM Tris, 40 mM 6-アミノカプロン酸, 20% メタノール）
TTBS（25 mM Tris-HCl pH7.6, 0.5 M NaCl, 0.05% Tween-20）
ブロッキングバッファー（5% スキムミルク in TTBS）
抗体(一次抗体 anti-PrP antibody、二次抗体)
ウエスタンブロット用検出試薬

実験の手順
1. 細胞から細胞溶解液作製
2. Proteinase K 処理と遠心分離
3. SDS-PAGE とウエスタンブロット

実験の詳細

1. 細胞から細胞溶解液作製

6 well プレートでプリオン持続感染細胞をコンフルエントの状態まで培養する。安全キャビネット内で培養液をアスピレーターで取り除く。冷 PBS をプレートの縁から 1 mL 加え、軽く撹拌、アスピレーターで PBS を取り除く。冷抽出バッファーを 500 µL 加え、細胞全体に行き渡るように軽く撹拌して細胞を溶解させる。1 分後、1.5 mL チューブに細胞溶解液を回収する。3,000 x g、10 分、4℃で遠心する。沈渣を取らないように上清を 1.5 mL チューブに回収する。

2. Proteinase K (PK)処理

回収した細胞溶解液 200 µl をセーフロックチューブに用意して 1 mg/mL の Proteinase K を 100 倍希釈して終濃度 10 µg/mL になるように加え、37℃、30 分で蛋白質分解反応を行う。チューブを氷上で冷却し、その後 0.1 M PMSF を 2 µL 加えて蛋白質分解反応を止める。その後、チューブを混和装置にセットして室温で 5 分間、溶液を混和する。そして 20,000 x g、20 分、4℃で遠心後、上清を取り除く。SDS-PAGE 用サンプルバッファーを 20 µL 添加して激しく撹拌する。5 分間煮沸して蛋白質を変性させる。

3. SDS-PAGE とウエスタンブロット

15% ポリアクリルアミドゲルを作製（または、プレキャストゲルを使用）し、10 µL のサンプルを注入して通常の SDS-PAGE を行う。

電気泳動後、セミドライトランスファー装置を用いて PVDF 膜に転写する。予め、PVDF 膜をメタノールに染み込ませ、その後、10 分以上精製水で浸透して、水に馴染ませる。また、3MM

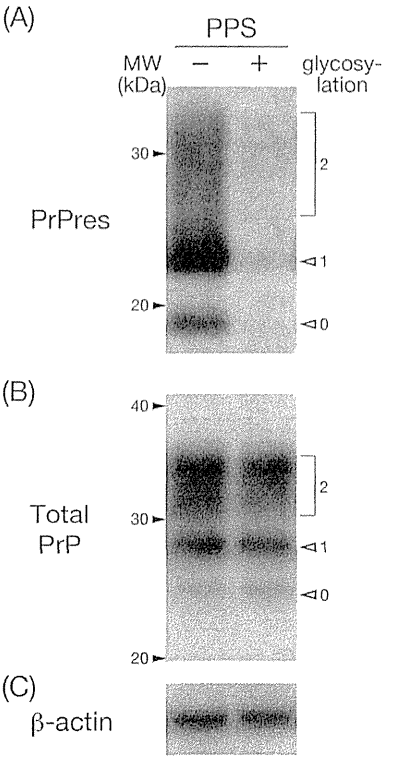


図 1：RML プリオン株を N2a 細胞に感染させた細胞 (ScN2a) (3) の PrP の検出と、抗プリオン作用を持つペントサンポリサルフェート (PPS) (4) の効果をみた結果。(A) PrPres の検出。レーン 1、PPS を培養液中に添加していない ScN2a 細胞；レーン 2、PPS を培養液中に添加した ScN2a 細胞。左の数字は MagicMark™ XP Western Protein Standard (Life Technologies 社) より判断した分子量サイズを示す。右の数字は PrP に結合している糖鎖の数を示す。(B) 全 PrP 蛋白質の検出。(C) 内因性コントロールとしての β-アクチンの検出。

紙 2 枚を陽極液 1 に、1 枚を陽極液 2 に、3 枚を陰極液に浸す。(下面が陽極のセミドライトランスファー装置では) 陽極液 1 を浸した 3MM 紙 2 枚をトランスファー装置の上に置く。その上に陽極液 2 を浸した 3MM 紙 1 枚を置く。さらに、精製水で平衡状態にさせた PVDF 膜を置く。そして、SDS-PAGE を行ったゲルを置く。最後に、陰極液を浸した 3MM 紙 3 枚を重ねる。3MM 紙や PVDF 膜、ゲルを重ねる際には、気泡が入らないように注意する。装置の準備が完了後、2mA/cm^2 の定電流で 40 分から 60 分、転写を行う。

蛋白質を転写した PVDF 膜は、ブロッキングバッファー中で 1 時間のブロッキングを行う。TTBS で 3 回洗浄したあと、1 次抗体 anti-PrP antibody を 1 時間反応させる。そして、TTBS で 3 回洗浄し、2 次抗体を 1 時間反応させる。その後、TTBS で 3 回洗浄し、検出試薬を用いて PrPres のシグナルを検出する (図 1A)。PrP は糖鎖結合部位が 2 箇所あるため、無糖鎖型 PrP、一糖鎖型 PrP、ブロードな二糖鎖型 PrP の 3 本のバンドが検出される。無糖鎖型 PrP は 20 kDa より少し小さく、一糖鎖型は 22 kDa、二糖鎖型は 28 kDa 付近にバンドが検出される。PK 処理をすると PrP の N 末端側は分解を受けて PK 耐性の C 末端が残るために、PK 未処理の PrP 全長に比べ小さい分子量になる (図 1A,B)。

工夫とコツ

細胞培養

当研究室ではOPTI-MEMに10% FBSを加えた溶液（6 well plateの場合3mL）を培養液として使用している。プリオン持続感染細胞の倍加時間は、細胞株の種類によって異なるが、おおよそ24時間である。細胞を3日間培養して蛋白質を回収する場合は、コンフルエントの細胞を8-10倍に希釈して約2 x 10^5 cells/wellを播いて培養する。3日後にはコンフルエントの状態に達し、約2 x 10^6 個の細胞が得られる。

細胞溶解液

PK処理を行うため、PKを阻害するようなセリンプロテアーゼインヒビターを加えてはならない。

蛋白質の定量

蛋白質の定量を行う場合は、細胞溶解液に界面活性剤が入っているためにBradford法は使用できない。当研究室ではLowry法を採用している。本条件では、1ウェル当たりコンフルエント状態の細胞からおよそ1 mgの総蛋白質量が得られる。後のSDS-PAGEでは、1レーンあたり200 µgの総蛋白質を使用してPK処理した量相当を添加すれば、PrPresの検出が可能である。

PrPresの遠心分離

組織からPrPresを検出する目的で組織溶解液に陰イオン性界面活性剤のサルコシルを使用するプロトコールがあるが、細胞溶解液にサルコシルを使用すると高分子の核酸がゲル状となり、PK処理を行った後のPrPresの分離が困難となる。本実験では、陰イオン性界面活性剤にデオキシコール酸ナトリウムを使用している。この界面活性剤を使用することで、高分子の核酸は糸くず状のまま除くことができる。また、PK処理後のPrPが容易に沈殿しやすくなり、一般的な遠心機で可能な20,000 x gの遠心力でPrPresを沈殿させることができる。

実験の詳細2では、遠心後の沈殿物が見えないので、上清を取り除く際に誤って沈殿物も取り除いてしまう可能性がある。そこで、遠心前の溶液に100倍希釈したglass milkを5 µL加えてから遠心することで、沈殿物を見やすくすることができる。

SDS-PAGE用サンプルバッファー

プリオン株によっては還元剤の2-メルカプトエタノールを加えないほうが検出しやすいことがあるので、PrPresの検出が弱い場合には、2-メルカプトエタノールのないサンプルバッファーを試してみるのも良い。

PVDF膜への転写

ポリアクリルアミドゲルの厚さによって転写時間を調節する。当研究室では、厚さが1.0 mmの場合は40分、1.5 mmの場合は60分で行っている。

ウエスタンブロット

当研究室では一次抗体に anti-PrP monoclonal antibody SAF83 (SPI-Bio 社)を 5,000 倍希釈で使用している。また、二次抗体は Anti-Mouse IgG (H+L), AP Conjugate (Promega 社) を 20,000 倍希釈で使用している。検出は CDP-*Star* detection reagent (GE Healthcare 社)を使用し、イメージアナライザー又は X 線フィルムで検出を行っている。

全 PrP と内因性コントロールの定量

PrPres の定量を行う際には全 PrP とインターナルコントロールを検出して、全 PrP の増減変化の確認とサンプル間の標準化を行うと良い。プリオン持続感染細胞の全 PrP に含まれる PrPres の割合は 100%ではなく、感染細胞の種類によるが、5-50%程度であるので、全 PrP を検出するためには、PrPres の検出で使用した細胞溶解液より少なくて良い。実験の詳細 1 で得られた細胞抽出液 40 µL に、5x サンプルバッファーを 10 µL 加え、5 分間煮沸して蛋白質を変性させる。その後は、実験の詳細 3 と同様に全 PrP を検出する(図 1B)。検出したメンブレンをストリッピングバッファー (2 M glycine, pH2.8[HCl で調整]) に浸して、室温、30 分の処理で抗体をはがす。新たにインターナルコントロール用の抗体を反応させて、検出を行う。当研究室では一次抗体に Anti-β-Actin monoclonal antibody (SIGMA 社)を 10,000 倍希釈で使用している。

PrP の糖鎖除去

プリオン蛋白質をアスパラギン型糖鎖を切断する酵素、ペプチド:N-グリカナーゼ (PNGase) で消化することがある。無糖鎖型、1 糖鎖型、2 糖鎖型のブロードな 3 本のプリオン蛋白質のバンドが、PNGase で糖鎖を切断することで無糖鎖型の 1 本のバンドになるため、より正確にプリオン蛋白質の量をサンプル間で比較検討できる。

プリオン持続感染細胞の入手と継代培養

日本や海外でプリオン持続感染細胞を使用している研究室から入手可能である。ただし、後述する安全対策を施す必要がある。また、入手直後に大量に細胞を増殖させて多数のチューブに細胞を凍結保存することを推奨する。プリオン持続感染細胞がプリオン (PrPres) を維持できる世代は無限ではなく、継代数が増えると細胞が PrPres を失う傾向にある。PrPres が何世代で消失するかは培養条件や細胞の種類によるが、細胞を解凍してから約 20 回以内の継代に留めるのが良い。プリオンが持続感染状態にあるかどうかは、本稿で紹介した方法で PrPres の存在を確認すると良い。また、細胞の形態を観察して異常が起きていないことを確認することも重要である。

脳組織の PrPres の検出

基本的には今回紹介した方法と同じような過程で脳内の PrPres を検出することができる。しかし、組織溶解液の組成は異なり、一般的に PK 濃度は高い。培養細胞に比べ、脳組織の PrPres の濃度は高いので、遠心して PrPres を濃縮しなくてもウエスタンブロットで検出が可能である。

実験の安全

動物に由来するプリオンの実験は、農林水産省公表の実験指針に従って、安全対策をとる。スクレイピープリオンはバイオセーフティレベル２に相当するので、本実験を行うには、そのレベルに対応できる実験室を使用し、安全キャビネットの中で実験を行う。プリオンに接触したチップ、チューブ等のプラスチック類は、135℃、30 分のオートクレーブ処理を行う。廃液については、SDS の濃度が 3%以上になるようにしてから、150℃、30 分のオートクレーブ処理を行う。詳細は「動物の伝達性海綿状脳症の実験指針（改正後）」に記載されている。

また SDS-PAGE のためのサンプルの加熱中に、誤ってチューブが開くのを阻止するために、チューブはセーフロックチューブを使用するのが望ましい。

参考文献

1) Prusiner, S.B., *Proc. Natl. Acad. Sci. USA.*, **95**, 13363-83 (1998)
2) Butler, D.A. et al., *J. Virol.*, **62**, 1558-64 (1988)
3) Race, R.E. et al., *J. Virol.*, **62** 2845-9 (1988)
4) Caughey, B. & Raymond, G.J., *J. Virol.*, **67** 643-50 (1993)
5) Kawasaki, Y. et al., *J. Virol.*, **81** 12889-98 (2007)
6) Hamanaka, T. et al., *Biochem. Biophys. Res. Commun.*, **405**, 285-90 (2011)
7) Nishizawa, K. et al., *J. Virol.*, **88**, 4083-99 (2014)
8) Kimura, T. et al., *FEBS Lett.*, **584**, 1193-8 (2010)
9) Safar, J. et al., *Nat. Med.*, **4**, 1157-65 (1998)
10) Timmes, A.G. et al., *PLoS One*, **8**, e71081 (2013)

総　説

[木材学会誌　Vol. 60, No. 3, p. 144-168 (2014)]

セルロース誘導体の置換位置制御と機能*1

上高原浩*2

Regiocontrolled Synthesis of Cellulose Derivatives and Their Functions*1

Hiroshi KAMITAKAHARA*2

This review focuses on synthesis methods of cellulose derivatives, in particular, those of cellulose ethers, from viewpoints of regiocontrolled introduction of protective and functional groups, published over roughly a quarter of a century. Influence of regioselective functional groups of cellulose on performance is discussed with particular focus on methylcellulose derivatives, 2-*O*-methyl, 3-*O*-methyl, 6-*O*-methyl, 2, 3-di-*O*-methyl, 2, 6-di-*O*-methyl, 3, 6-di-*O*-methyl, and 2, 3, 6-tri-*O*-methyl celluloses. Synthesis methods for tri-*O*-alkyl celluloses (carbon numbers of alkyl chain = 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 14 and 18), and alternatingly 2-*O*- and 6-*O*-methylated celluloses are also summarized. In addition, regioselectively functionalized cellulose esters are described. Moreover, synthesis methods of cellulosic block copolymers with other polymer blocks and diblock cellulose ethers with regioselective functionalization patterns are summarized. New insights into structure-property relationships of methylcellulose with blocky structure to form thermoresponsive hydrogels are described in detail.

Keywords: cellulose, cellulose derivatives, regioselective functionalization, methylcellulose, diblock copolymer.

本総説では，この四半世紀ほどの間に報告されたセルロース誘導体，特にセルロースエーテル誘導体，および若干のセルロースエステル誘導体の実験室レベルでの合成法について，位置選択的な保護基/官能基導入の観点からまとめている。具体的には，トリ-*O*-アルキルセルロースの合成法，交互置換 *O*-メチルセルロースの酵素合成法，セルロース系ブロックコポリマーの合成法を挙げる。次いで，セルロース誘導体の置換位置の違いがその機能に与える影響について，メチルセルロースを1例として論じる。さらに，新しいブロック的メチル化セルロースからなる熱応答性ヒドロゲルの構造-物性相関について最近の知見をまとめている。

1. はじめに

21世紀に入り，環境負荷の低いセルロースは工業製品を製造するための原材料としてその重要性が再認識されている。歴史を遡れば Hermann Staudinger 以来セルロースは高分子化学の中心であったものの，いつしか石油化学を基盤とする合成高分子へと時代の趨勢は移り変わっていった。しかしながら，近年，再生可能資源であるセルロースへの研究回帰が見られ，1990年代以降，セルロースの酵素合成法[1]やカチオン開環重合による化学合成法[2]の確立，新しい水系セルロース溶媒[3]の発見，セルロースの水酸基に対する優れた保護基[4]の開発などが続いている。世界的にバイオマス研究が隆盛している状況の

*1 Received December 21, 2013; accepted January 22, 2014.

*2 京都大学大学院農学研究科森林科学専攻　Graduate School of Agriculture, Kyoto University, Kyoto 606-8502, Japan
Corresponding author: H. Kamitakahara (hkamitan@kais.kyoto-u.ac.jp)